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STROMAL-EPITHELIAL INTERACTIONS MODULATE CROSS TALK BETWEEN PROLACTIN RECEPTOR AND HER2/NEU IN BREAST CANCER

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STROMAL-EPITHELIAL INTERACTIONS MODULATE CROSS TALK BETWEEN
PROLACTIN RECEPTOR AND HER2/NEU IN BREAST CANCER

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ABSTRACT

The tumor microenvironment is a crucial factor in breast tumorigenesis. Tumor epithelial cells maintain 3D structure in tumor stroma and they interact with soluble factors secreted by stromal cells such as cancer associated fibroblasts (CAFs) or directly with the extracellular matrix (ECM). Recent studies have shown that the hormone prolactin (PRL) promotes the proliferation and survival of breast cancer cells in part via the transactivation of human epidermal growth factor receptor 2 (HER2), also known as Neu in rodents. A PRL receptor (PRLR) antagonist, G129R, has been demonstrated not only to be able to directly inhibit PRLR activation but also indirectly inhibits the tyrosine phosphorylation of HER2 (p-HER2) in human breast cancer cell lines. However, there is an obvious discrepancy related to the inhibitory effects of G129R on p-HER2 between the monolayer cell culture system and *in vivo*.

In this dissertation, I investigated the potential mechanisms by which tumor stroma exert upon the cross talk between PRLR and HER2/Neu. To compare drug response to G129R between tumors and primary cultured cells, mammary tumors were resected and cultured as small tumor chunks (≈ 3 mm^3) or were cultured in monolayer. G129R reduced p-Neu in a dose-dependent manner (IC$_{50}$ ≈ 10 µg/ml) in tumor chunks, but had no significant effect upon primary tumor epithelial cells grown in monolayer. Similar to that observed in mouse tumor chunks, direct co-culture of mouse tumor epithelial cells with CAFs restored the response of epithelial cells to G129R. The addition of PRL, as expected, induced p-Neu in both the tumor chunk and co-culture

models. The inhibitory effect of G129R was absent when CAFs were physically separated from mouse tumor epithelial cells using a transwell system, or when CAFs were replaced with normal fibroblasts in direct co-culture with human or mouse tumor epithelial cells. In human breast cancer cell lines, the activation of HER2 was reduced by G129R when co-cultured with mouse or human CAFs. Furthermore, 3D culture of just mouse or human tumor epithelial cells with ECM components restored the same response to both G129R and PRL, suggesting that ECM components or 3D cell structure at least partially contributes to the cross talk between HER2/Neu and PRLR in breast cancer. Finally, G129R was tested in *ex vivo* and *in vivo* models. I demonstrated that G129R had an additive inhibitory effect on p-Neu in tumor chunks when combined with lapatinib, a HER1 and HER2/Neu tyrosine kinase inhibitor. Also, it reduced tyrosine phosphorylation of Neu (p-Neu) in primary mammary tumors of mice in a time- and dose-dependent manner.

In conclusion, tumor stroma play a critical role in modulating the cross talk between PRLR and HER2/Neu in both human and mouse models of breast cancer. The inhibitory effects of G129R on p-HER2/Neu are dependent, at least in part, upon interactions of tumor epithelium with stroma.

DEDICATION

I dedicate this dissertation to my family. Their love and encouragement along this journey have been the most precious. And the wisdom and courage they have brought me will become an invaluable treasure for a lifetime.

ACKNOWLEDGMENTS

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ABBREVIATIONS

2D	Two Dimensional
3D	Three Dimensional
AKT/PKB	Protein Kinase B
Arg	Arginine
Bcl2	B-cell lymphoma 2
CAF	Cancer Associated Fibroblast
cAMP	cyclic Adenosine Monophosphate
CCL5	Chemokine (C-C motif) Ligand 5
CDK	Cyclin-Dependant Kinase
CSF-1	Colony-Stimulating Factor-1
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen Receptor
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FGF	Fibroplast Growth Factor
FVB/N	Inbred strain of mice for the production of transgenic mice
G129R	Human prolactin receptor antagonist

GH	Growth Hormone
HER2	Human Epidermal Growth Factor Receptor-2
HGF	Hepatocyte Growth Factor
HIF1	Hypoxia-Inducible Factor 1
HRG	Heregulin
IGF-1	Insulin-like Growth Factor-1
IL-6	Interleukin-6
JAK2	Janus Kinase 2
MAPK	Mitogen-Activating Protein Kinase
MCNeuA	Epithelial cell line derived from tumor in MMTV-*neu* mice
MMP	Matrix Metalloproteinase
MMTV	Mouse Mammary Tumor Virus
mRNA	Messenger Ribonucleic Acid
MTOR	Mammalian Target Of Rapamycin
N202Fb3	Fibroblast cell line derived from tumor in MMTV- *neu* mice
NRG	Neuregulin
PBS	Phosphate -Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
p-HER2	Tyrosine phosphorylated HER2
PI3K	Phosphatidylinositol 3-Kinase
PINCH	Particularly Interesting New Cysteine-Histidine

PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PKA	Protein Kinase A
PLC	Phospholipase C
PMSF	Phenylmethylsulphonyl Fluoride
PR	Progesterone Receptor
PRL	Prolactin
PTEN	Phosphatase and Tensin homolog
Rb	Retinoblastoma
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SDF-1	Stromal cell-Derived Factor-1
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SH	Src Homology
STAT	Signal Transducers and Activators of Transcription
TBS-T	Tris-Buffer Saline-Tween 20
TGF-α	Transforming Growth Factor Alpha
TGF-β	Transforming Growth Factor Beta
TK	Tyrosine Kinase
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor
α-SMA	Alpha Smooth Muscle Actin

CHAPTER ONE

INTRODUCTION

Cancer

Cancer is known as malignant neoplasm, the growth of which is incoordinate with the surrounding normal tissues and that persists in the absence of the inciting stimulus. There are more than 100 distinct types of cancer, and subtypes of tumors can be found within specific organs [197].

In 2008, approximately 12.7 million cancers were diagnosed (excluding non-melanoma skin cancers and other non-invasive cancers) and 7.6 million people died of cancer worldwide [147]. Cancers as a group account for approximately 13% of all deaths each year with the most common being: lung cancer (1.4 million deaths), stomach cancer (740,000 deaths), liver cancer (700,000 deaths), colorectal cancer (610,000 deaths), and breast cancer (460,000 deaths). This makes invasive cancer the leading cause of death in the developed world and the second leading cause of death in the developing world [294].

Only 5-10% of all cancer cases can be attributed to genetic defects, whereas the remaining 90-95% have their roots in the environment and lifestyle [294]. These risk factors include cigarette smoking, diet (fried foods, red meat), alcohol, sun exposure, environmental pollutants, infections, stress, obesity, and physical inactivity [294]. The evidence indicates that of all cancer-related deaths, almost 25-30% are due to tobacco, as many as 30-35% are linked to diet, about 15-20% are due to infections, and the remaining

percentage are due to other factors like radiation, stress, physical activity, environmental pollutants [294]. All the risk factors directly damage genes or combine with existing genetic faults within cells to cause the disease [161].

Hallmarks of Cancer

Cancer arises from normal tissues and it is fundamentally a disease of failure of regulation of tissue growth [291]. Histopathology and genomic studies have provided evidence that cancer progression is a multi-step process in which a somatic cell first undergoes an initiating event (e.g. environmental damage) and then a second or promoting event such as loss of heterogeneity. The accumulation of genetic alterations is thought to drive cancer progression [291]. A carcinogen or mutagen, for instance from alcohol, when drunk in sufficient quantity and duration may act to form an unwanted bond on DNA and potentially mutating a gene [125]. Also, many cancers will develop as a result of a chronic inflammatory state due to infections [195].

Weinberg *et al*. suggested that all of the cancer cell genotypes were a manifestation of six essential alterations in cell physiology that collectively dictated tumor growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory
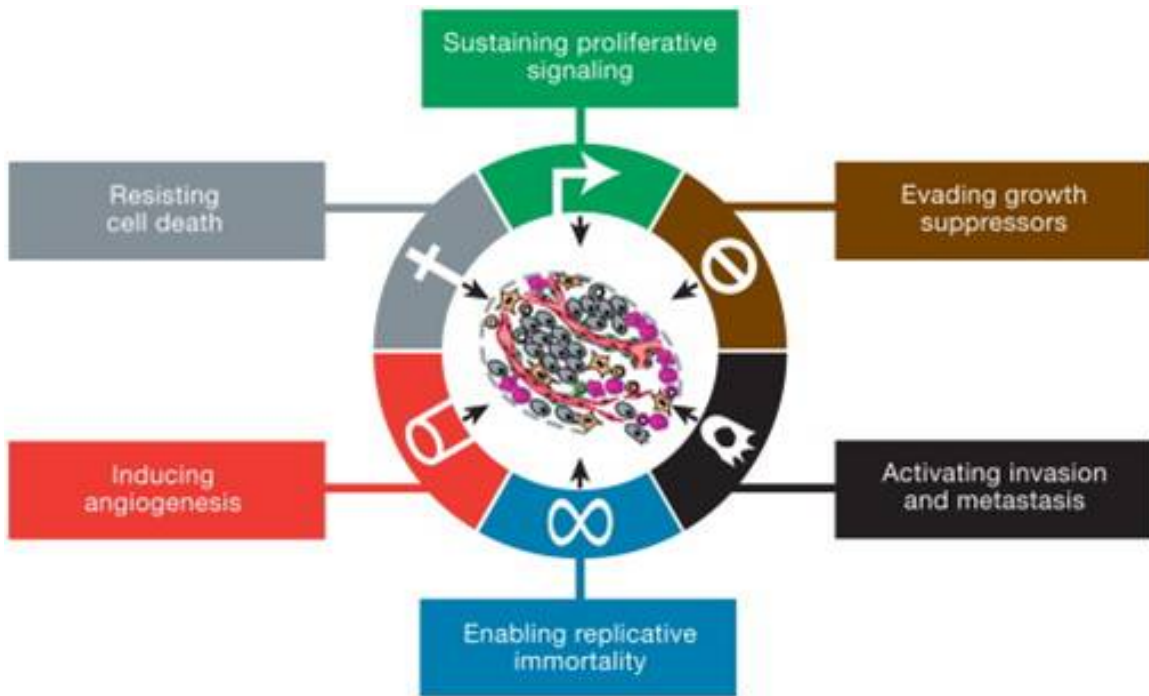
Fig.1.1   The hallmarks of cancer

This illustration encompasses the six hallmark capabilities [126].

(anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Fig.1.1). They proposed that these six capabilities were shared in common by most and perhaps all types of human tumors [125].

Recently, Weinberg *et al*. introduced two additional hallmarks of cancer involved in the pathogenesis of cancers. One involves the capability to modify, or reprogram, cellular metabolism in order to most effectively support neoplastic proliferation. The second allows cancer cells to evade immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells [126].

Sustaining proliferative signaling

Normal tissues control the production and release of growth-promoting signals through the cell growth and division cycle, thereby ensuring a homeostasis of cell number and maintenance of normal tissue architecture and function. Cancer cells, due to the disruption in these signals, transform into cells under their own control. The enabling signals are conveyed in large part by growth factors that bind cell-surface receptors, typically containing intracellular tyrosine kinase domains. The latter subsequently transmits signals via branched intracellular signaling pathways that regulate progression through the cell cycle as well as cell growth; often these signals influence yet other cell-biological properties, such as cell survival and energy metabolism [126].

Somatic mutations activate additional downstream pathways. DNA sequencing analyses of cancer cell genomes have revealed somatic mutations in certain human tumors that predict constitutive activation of signaling pathways usually triggered by activated growth factor receptors. Approximately 40% of human melanomas contain activating mutations affecting the structure of the B-RAF protein, resulting in constitutive signaling through the RAF to mitogen activated protein kinase (MAPK) pathway [67]. Similarly, mutations in the catalytic subunit of phosphoinositide 3-kinase (PI3K) isoforms are being detected in an array of tumor types, which serve to overactivate the PI3K signaling pathway, including its key AKT/PKB signal transducer [148, 311]. The advantages to tumor cells of activating upstream (receptor) versus downstream (transducer) signaling remain obscure, as does the significance of cross talk between the multiple pathways from growth factor receptors [126].

Negative-feedback mechanisms in proliferative signaling are disrupted to tumors. Recent results have highlighted the importance of negative feedback that normally operate to suppress various types of signaling and thereby ensure homeostatic regulation of the intracellular signals [42, 292]. Defects in these feedback mechanisms are in turn enhancing proliferative signaling. This type of regulation involves the RAS oncoprotein: the oncogenic effects of RAS result from the compromised RAS GTPase activity, which operates as an intrinsic negative-feedback mechanism that normally ensures active signal transmission is short-term only. A prominent example involves the PTEN phosphatase, which counteracts PI3K by degrading its product, phosphatidylinositol trisphosphate

(PIP3). Yet another example involves the mTOR kinase, a coordinator of cell growth and metabolism that lies both upstream and downstream of the PI3K pathway [216, 266].

Evading growth suppressors

Cancer cells must circumvent programs that negatively regulate cell proliferation; many of these programs depend on the actions of tumor suppressor genes. Tumor suppressors operate in various ways to limit cell growth and proliferation. The two most common tumor suppressors encode the retinoblastoma-associated protein (RB) and tumor protein 53 (TP53). The RB protein integrates signals from diverse extracellular and intracellular sources and, in response, decides whether or not a cell should proceed through its growth-and-division cycle [41]. Cancer cells with defects in RB pathway function are thus missing the services of a critical gatekeeper of cell-cycle progression whose absence permits persistent cell proliferation. Whereas RB transduces growth-inhibitory signals that originate largely outside of the cell, TP53 receives inputs from stress and abnormality sensors that function within the cell's intracellular operating systems: if the degree of damage to the genome is excessive, or if the levels of nucleotide, growth-promoting signals, glucose, or oxygenation are abnormal, TP53 can pause further cell-cycle progression until these conditions have been normalized [126].

Apoptosis, the programmed cell death is triggered in response to various physiologic stresses that cancer cells experience during the course of carcinogenesis. It is attenuated in those tumors that succeed in progressing to states of high-grade malignancy and resistance to therapy [2]. The apoptotic program is composed of both upstream

regulators such as Fas ligand/Fas receptor and downstream effector components like caspases 8 and 9, which proceeds to initiate a cascade of proteolysis, in which the cell is progressively disassembled and then consumed, both by its neighbors and by phagocytic cells [2]. Tumor cells develop a variety of strategies to limit or circumvent apoptosis. Most common is the loss of TP53 tumor suppressor function, which eliminates this critical damage sensor from the apoptotic program. Alternatively, tumors may achieve similar ends by increasing expression of anti-apoptotic regulators (Bcl-2) or of survival signals (IGF1/2), by down-regulating pro-apoptotic factors (Bax, Bim), or by interrupting the extrinsic ligand-induced death pathway. The multiplicity of apoptosis-avoiding mechanisms presumably reflects the diversity of apoptosis-inducing signals that cancer cell populations encounter during their evolution to the malignant state [126].

Autophagy mediates both tumor cell survival and death. Autophagy represents an important physiologic response that normally operates at low levels in cells but can be strongly induced in certain states of cellular stress, the most obvious of which is nutrient deficiency [182, 199]. The autophagic program enables cells to break down cellular organelles, such as ribosomes and mitochondria, allowing the resulting catabolites to be recycled and thus used for biosynthesis and energy metabolism. As a result, low molecular-weight metabolites are generated to support survival in the stressed, nutrient-limited environments experienced by many cancer cells. Like apoptosis, the autophagy machinery has both regulatory and effector components [199]. They are proteins that mediate autophagosome formation and delivery to lysosomes. The signaling pathways involve the PI3K, AKT, and mTOR kinases [199]. For instance, mice bearing inactivated

alleles of the Beclin-1 gene or of certain other components of the autophagy machinery exhibit increased susceptibility to cancer [199]. These results suggest that induction of autophagy can serve as a barrier to tumorigenesis that may operate independently of or in concert with apoptosis [293].

Necrosis has pro-inflammatory and tumor-promoting potential. In contrast to apoptosis, in which a dying cell contracts into an almost-invisible corpse, necrotic cells become bloated and explode, releasing their contents into the local tissue. Necrotic cell death releases pro-inflammatory signals into the surrounding tissue. As a consequence, necrotic cells can activate the immune system [104]. Evidence indicates that immune inflammatory cells can be actively tumor promoting, given that such cells are capable of inducing angiogenesis, cancer cell proliferation, and invasiveness. Additionally, necrotic cells can release bioactive regulatory factors, such as IL-1, which can directly stimulate neighboring cells to proliferate, with the potential to facilitate neoplastic progression [119]. Consequently, necrotic cell death, while seemingly beneficial in counterbalancing cancer-associated hyperproliferation, may ultimately do more damage than good [126].

Enabling replicative immortality

Multiple lines of evidence indicate that telomeres protecting the ends of chromosomes are involved in the capability for unlimited proliferation [240]. The telomeres, composed of multiple tandem hexanucleotide repeats, shorten progressively in non-immortalized cells, eventually losing the ability to protect the ends of chromosomal DNAs from end-to-end fusions; such fusions generate unstable chromosomes whose

resolution results in the loss of cell viability. Accordingly, the length of telomeric DNA in a cell dictates how many successive cell generations its progeny can pass through before telomeres are largely eroded and have consequently lost their protective functions, triggering cell death. Telomerase, the specialized DNA polymerase that adds telomere repeat segments to the ends of telomeric DNA, is almost absent in non-immortalized cells but expressed at significant levels in 90% of spontaneously immortalized cells, including human cancer cells. By extending telomeric DNA, telomerase is able to counter the progressive telomere erosion. The presence of telomerase activity, either in spontaneously immortalized cells or in the context of cells engineered to express the enzyme, is correlated with a resistance to induction of cell senescence. The eventual immortalization of cells that proceed to form tumors has been attributed to their ability to maintain telomeric DNA at lengths sufficient to avoid triggering senescence, achieved most commonly by up-regulating expression of telomerase or, less frequently, via an alternative recombination-based telomere maintenance mechanism [126].

Inducing angiogenesis

Like normal tissues, tumors require sustenance in the form of nutrients and oxygen as well as an ability to evacuate metabolic wastes and carbon dioxide. The tumor-associated neovasculature, generated by the process of angiogenesis, addresses these needs. During tumor progression, an angiogenic switch is almost always activated, causing normal vasculature to continually generate new vessels that help sustain expanding neoplastic growths [124].

The blood vessels produced within tumors by chronically activated angiogenesis are typically aberrant: tumor neovasculature is marked by precocious capillary sprouting, convoluted and excessive vessel branching, distorted and enlarged vessels, erratic blood flow, microhemorrhages, leakiness, and abnormal levels of endothelial cell proliferation and apoptosis [14, 210].

Observations suggest an initial angiogenic switch takes place during tumor development followed by a variable intensity of ongoing neovascularization [13]. Of note, the switching mechanism can vary in its form, even though the net result is a common inductive signal (e.g., VEGF). In some tumors, dominant oncogenes operating within tumor cells, such as RAS and Myc, can up-regulate expression of angiogenic factors, whereas in others, such signals are produced indirectly by immune inflammatory cells, such as macrophages, neutrophils, mast cells, and myeloid progenitors. Additionally, pericyte coverage is also important for the maintenance of a functional tumor neovasculature [24].

Activating invasion and metastasis

The multistep process of invasion and metastasis has been described as a sequence of many steps, often termed the invasion-metastasis cascade [268]. It is a succession of cell-biologic changes, beginning with local invasion, then intravasation by cancer cells into nearby blood and lymphatic vessels, transit of cancer cells through the lymphatic and hematogenous systems, followed by escape of cancer cells from the lumina of such vessels into the parenchyma of distant tissues (extravasation), the

formation of small nodules of cancer cells (micrometastases), and finally the growth of micrometastatic lesions into macroscopic tumors, this last step being termed "colonization" [126]. Thus, overall, metastasis can be broken down into two major phases: the physical dissemination of cancer cells from the primary tumor to distant tissues, and the adaptation of these cells to foreign tissue microenvironments that results in successful colonization, i.e., the growth of micrometastases into macroscopic tumors [126].

The down-regulation and occasional mutational inactivation of E-cadherin in human carcinomas provided strong support for its role as a key suppressor of this hallmark capability [28]. A developmental regulatory program, referred to as the epithelial-mesenchymal transition(EMT), has become implicated as a means by which transformed epithelial cells can acquire the abilities to invade, to resist apoptosis, and to disseminate [166, 230]. A set of transcriptional factors, including Snail, Slug, Twist, and Zeb1/2, participate in the EMT and related migratory processes during embryogenesis; most were initially identified by developmental genetics. These transcriptional regulators are expressed in various combinations in a number of malignant tumor types and have been shown in experimental models of carcinoma formation to be important for programming invasion [307].

Furthermore, cross talk between cancer cells and cells of the tumor stroma is also involved in the acquired capability for invasive growth and metastasis [84]. For example, mesenchymal stem cells (MSCs) present in the tumor stroma have been found to secrete

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CCL5 in response to signals released by cancer cells; CCL5 then acts reciprocally on the cancer cells to stimulate invasive behavior [155]. Macrophages at the tumor stroma can foster local invasion by supplying matrix-degrading enzymes such as metalloproteinases and cysteine cathepsin proteases [158]. And in an experimental model of metastatic breast cancer, tumor-associated macrophages (TAMs) supply epidermal growth factor (EGF) to breast cancer cells, while the cancer cells reciprocally stimulate the macrophages with CSF-1; their concerted interactions facilitate invasion into the circulatory system and metastatic dissemination of the cancer cells [233].

An emerging hallmark: reprogramming energy metabolism

The chronic and uncontrolled cell proliferation in cancer involves not only deregulated control of cell proliferation but also corresponding adjustments of energy metabolism in order to fuel cell growth and division. Otto Warburg first observed an anomalous characteristic of cancer cell energy metabolism: even in the presence of oxygen, cancer cells can reprogram their glucose metabolism, and thus their energy production, by limiting their energy metabolism largely to glycolysis, leading to a state that has been termed "aerobic glycolysis" [289-290].

Glycolytic fueling has been shown to be associated with activated oncogenes (e.g., RAS, MYC) and mutant tumor suppressors (e.g., TP53), whose alterations in tumor cells have been selected primarily for their benefits in cell proliferation, avoidance of cytostatic controls, and attenuation of apoptosis [71]. This reliance on glycolysis can be further accentuated under the hypoxic conditions that operate within many tumors: the

hypoxia response system acts to up-regulate glucose transporters and multiple enzymes of the glycolytic pathway [251]. Thus, both the RAS and hypoxia can independently increase the levels of the HIF1α and HIF2α transcription factors, which in turn up-regulate glycolysis [252].

Altered energy metabolism is proving to be as widespread in cancer cells as many of the other cancer-associated traits that have been accepted as hallmarks of cancer. This realization raises the question of whether deregulating cellular energy metabolism is therefore a core hallmark capability of cancer cells that is as fundamental as the six well-established core hallmarks [127].

Another emerging hallmark: evading immune destruction

A second, still-unresolved issue surrounding tumor formation involves the role that the immune system plays in resisting or eradicating formation and progression of tumors, late-stage tumors, and micrometastases [126].

The role of defective immunological monitoring of tumors is validated by the increases of certain cancers in immunocompromised individuals [281]. An increasing body of evidence suggests that the immune system operates as a significant barrier to tumor formation and progression.

When immunedeficient mice were assessed for the development of carcinogen-induced tumors, it was observed that tumors arose more frequently and/or grew more rapidly in the immunodeficient mice relative to immunocompetent controls [270]. In

addition, transplantation experiments have shown that cancer cells that originally arose in immunodeficient mice are often inefficient at initiating secondary tumors in syngeneic immunocompetent hosts, whereas cancer cells from tumors arising in immunocompetent mice are equally efficient at initiating transplanted tumors in both types of hosts [160]. Clinical epidemiology also supports the existence of anti-tumoral immune responses in some forms of human cancer [33, 93]. For example, patients with colon and ovarian tumors that are heavily infiltrated with CTLs and NK cells have a better prognosis than those that lack such abundant killer lymphocytes [212].

On the other hand, highly immunogenic cancer cells may well evade immune destruction by disabling components of the immune system. For example, cancer cells may paralyze infiltrating CTLs and NK cells, by secreting TGF-β or other immunosuppressive factors [308]. More subtle mechanisms operate through the recruitment of inflammatory cells that are actively immunosuppressive, including regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs). Both can suppress the actions of cytotoxic lymphocytes [203].

Breast Cancer

Breast cancer (malignant breast neoplasm) is a type of cancer originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk [244]. In developed countries, breast cancer is the most commonly diagnosed malignancy in women and is the second leading cause of cancer death. Worldwide, breast cancer comprises 22.9% of all cancers (excluding non-

melanoma skin cancers) in women. In 2006, estimated new breast cancers in the United States were 212,920 and deaths, 40,600 [116]. In 2008, breast cancer caused 458,503 deaths worldwide (13.7% of cancer deaths in women) [144]. Many women with breast cancer do not have any of the known risk factors [116]. The relative risk factors for breast cancer include female sex [111], age, lack of childbearing or breastfeeding, higher hormone levels race, economic status and dietary iodine deficiency [303]. Breast cancer, like other cancers, occurs because of an interaction between the environment and defective gene, such as *p53*, *BRCA1* and *BRCA2*. These mutations in breast cancer allow uncontrolled cell division, lack of attachment, and metastasis to distant organs [81].

Most breast cancers are derived from the epithelium lining the ducts or lobules. The *in situ* carcinomas of the breast are classified as ductal (DCIS), lobular (LCIS), or Paget's disease of the nipple [116]. They are growth of low grade cancerous or precancerous cells within a particular tissue compartment such as the mammary duct without invasion of the surrounding tissue. However, most invasive breast cancers are adenocarcinomas, with invasive ductal carcinoma being the commonest (80%) and invasive lobular carcinoma occurring approximately 10% of the time [116].

Several important receptors are expressed on the surface of breast cancer cells, in their cytoplasm and in the nucleus. Hormones bind to receptors and initiate transcriptional changes in the cells. Clinicians often check three critical receptors: estrogen receptor (ER), progesterone receptor (PR), and HER2/Neu. ERs are expressed in 60% of all breast cancers and indicate that cancer cells depend on estrogen for their

growth, so they can be responsive to ER modulator (e.g. tamoxifen). This type of cancers generally has a better prognosis [197]. HER2-positive breast cancer have a worse prognosis, but can respond to drugs such as the monoclonal antibody trastuzumab (in combination with conventional chemotherapy), and this has improved the prognosis significantly [262]. Cells with none of these receptors are called basal-like or triple negative.

In addition to surgery and radiation therapy, there are currently three main groups of medications used for breast cancer treatment: hormonal manipulation, chemotherapy, and monoclonal antibodies. ER-positive cancers can be treated with ER antagonist, e.g. tamoxifen (Nolvadex), or alternatively aromatase inhibitor, e.g. anastrozole (Arimidex) or letrozole (Femara). Chemotherapy is predominately used for stage 2-4 disease, being particularly beneficial in ER-negative breast cancer. Anthracyclines and taxanes are the two most active drug classes against breast cancer. Many patients have often received these agents in the adjuvant setting. Capecitabine is used when the disease has recurred or progressed after anthracyclines and taxanes. The other active drugs include cytoxan, methotrexate, vinorelbine, gemcitabine, oral etoposide, and irinotecan. Trastuzumab (Herceptin), a monoclonal antibody to HER2, has improved the 5 year disease free survival of stage 1–3 HER2-positive breast cancers to about 87% (overall survival 95%) [116].

Tumor Microenvironment and 3D Structure

Tumor microenvironment is initially recognized in chronic inflammatory state. Many cancers will develop as a result of a chronic inflammatory state due to infections [171]. This is commonly seen with Hepatitis B and C, which can be a harbinger for hepatocellular carcinoma [171]. Gastric infection from *Helicobacter pylori*, will increase gastric cancer risk by 75%, and is the second most common type of cancer globally [171]. A few other clinical examples illustrating the association of chronic inflammation and increased cancer risk include: inflammatory bowel disease (ulcerative colitis, Crohn's disease) and colon cancer; cervical infection (human papillomavirus) and cervical cancer, and chronic reflux esophagitis resulting in Barrett's esophagus that is high risk for esophageal carcinoma [61]. In all cases, these chronic inflammatory conditions help to establish a tumor microenvironment full of deranged proliferative signaling networks, which is largely orchestrated by inflammatory cells and is an indispensable participant in the neoplastic process [61].

Several types of stromal cells such as cancer-associated fibroblasts (CAFs), endothelial cells, inflammatory cells, and extracellular matrix (ECM) collectively create the microenvironment for epithelial cells [32]. It is the niche of developing cross talk between different cells types. Critical stromal elements such as CAFs provide an essential communication network via secretion of growth factors and chemokines, inducing ECM alteration to generate additional carcinogenic signals enhancing cancer cell proliferation [152]. In cancer invasion, tumor cells secrete a variety of proteins that include growth factors and ECM-degrading proteinases to degrade the matrix and its

component adhesion molecules. The matrix degradation takes place in a region close to the tumor cell surface, where the amount of the active degradative enzymes outbalances the natural proteinase inhibitors present in the matrix or that secreted by normal cells [128]. Proteins secreted by tumor cell into the ECM microenvironment are therefore involved in cell adhesion, motility, intercellular communication and invasion [128]. Also, the stromal cells induce the requisite transcription programs allowing the necessary mesenchymal phenotypes to invade distant tissues and establish a new environment. The cancer cells then shut down the transcription factor programs and reconvert from mesenchymal to epithelial cells, thus recreating themselves from the core of primary tumor cells [291].

Fibroblasts

Fibroblasts were first described in the late 19th century, based on their location and their microscopic appearance. As a member in connective-tissue family, they are dispersed in connective tissue throughout the body, where they secrete a non-rigid ECM [201].

The primary function of fibroblasts is wound healing. When a tissue is injured, the fibroblasts nearby proliferate, migrate into the wound, and generate ECM to serve as a scaffold for other cells, which helps to isolate and repair the damaged tissue [6]. Fibroblasts synthesize many of the constituents of the fibrillar ECM such as type I, type III and type V collagen, and fibronectin [238, 275]. They also contribute to the formation of basement membranes by secreting type IV collagen and laminin [48]. As the principal

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source of ECM constituents, fibroblasts are considered the main mediators of scar formation and tissue fibrosis.

Additionally, fibroblasts are important in maintaining the homeostasis of adjacent epithelia through the secretion of growth factors and direct mesenchymal–epithelial cell interactions [296]. The latter may lead to epithelial-mesenchymal transition (EMT), a crucial stage involved in cancer cell metastasis. Fibroblasts are also an important source of ECM-degrading proteases such as matrix metalloproteinases (MMPs), which highlights their crucial role in maintaining an ECM homeostasis by regulating ECM turnover [48].

Fibroblasts need to be activated in wound healing. Inactive fibroblasts, which are also called fibrocytes, are smaller and spindle shaped. They have a reduced rough endoplasmic reticulum. On the other hand, fibroblasts isolated from the site of a healing wound or from fibrotic tissue secrete higher levels of normal ECM constituents and proliferate more than their normal counterparts isolated from healthy organs [46, 207]. Such increased activity is referred to as 'activation' [46]. Once the wound is repaired, the number of activated fibroblasts decreases significantly and the resting phenotype is thought to be restored [275]. It remains unknown whether the activated fibroblasts revert to a resting phenotype, or whether they undergo apoptosis followed by the repopulation of that particular region of the tissue by resting fibroblasts from the adjacent tissue [275].

Carcinoma-associated fibroblasts

It has been studied since 1970 that fibroblasts within the tumor stroma acquire a modified phenotype, similar to fibroblasts associated with wound healing [82]. Therefore, cancer is frequently compared to a wound that never heals. This is partially due to the perpetually activated fibroblasts at the site of the tumor. This subpopulation of fibroblasts in cancer is called cancer-associated fibroblasts (CAFs), commonly identified by their expression of α-smooth-muscle actin [21]. In breast carcinomas, about 80% of stromal fibroblasts acquire this activated phenotype [243]. It is becoming clear that CAFs are important promoters of tumor growth and progression [205].

Local fibroblasts or fibroblast precursors, stimulated by members of the PDGF or TGF-β family, have generally been considered as the major source of CAFs [152]. Recent studies discovered additional cellular sources of CAFs such as bone marrow-derived cells, malignant epithelial cells, and endothelial cells [39, 152, 312].

CAFs are an established source of classical growth factors known to possess a tumor-promoting role, for example EGF; TGF-β or HGF [152]. A pro-metastasis effect was demonstrated in experiments where different fibroblasts were co-injected with pancreatic cancer cells [142]. Studies also suggested cancer cells and CAFs express complementary metabolic pathways which facilitate CAFs to remove toxic metabolites and to buffer the acidity generated by cancer cells, thereby exerting a metabolic pro-tumorigenic effect [170]. CAFs reduced cancer cells sensitivity to chemotherapy in co-cultured or cancer cells grown in fibroblast-conditioned medium [142]. Similar findings

have been made in breast cancer models where tamoxifen sensitivity was affected by co-cultured fibroblasts [202]. VEGF derived from CAFs are important for tumor angiogenesis [80]. PDGF-dependent CAFs in a genetic model of cervix cancer also produced FGF which significantly contributed to tumor angiogenesis [227]. CXCL12 produced by breast CAFs contributes to the recruitment of bone marrow-derived endothelial precursor cells [218]. Finally, CXCL14, which is consistently up-regulated in human prostate CAFs, increased tumor content of macrophages, and also stimulated tumor growth [12]. In current therapeutic strategy CAFs-secreted tumorigenic molecules and the source of CAFs are the two primary targets. For instance, targeting stromal PDGF receptors in tumors increased tumor drug uptake [226]. In another study, inhibition of stromal PDGF receptors induced anti-tumoral effects in models of cervix and colorectal cancer [162, 227]. Targeting CXCL14 is another potential therapeutic approach for its role in CAFs stimulation [309].

3D cellular structure

The mammary gland, like many glandular organs, is embedded in stroma, which includes ECM. ECM not only provides structural support but also signaling cues via transmembrane receptors, directing cytoskeletal and chromatin organization to maintain tissue integrity [34]. It was shown that collagen gels, which provide a 3D scaffold, allow epithelial cells of various tissues and origins to maintain some of their tissue structure and differentiated functions [87].

The behavior of invasive carcinomas (e.g. breast, prostate, colon.) in humans can be very varied, in that they can metastasize rapidly in one case or take many years to become invasive in another [259]. These observations emphasize the dynamic and progressive nature of cancer in humans [159]. Heterologous 3D model systems allow cells to grow in a niche with similar nature. This system reflects the distinct invasive behavior of human tumor cells, mimics the tumor–stromal cell interactions of human carcinomas and allows for systemic investigation into the multiple unknown regulatory feedback mechanisms between tumor and stromal cells in a well-defined 3D environment [224].

The common strategy to construct a 3D system is to isolate cells in tissue culture and then implant them in a 3D matrix scaffold as either single cells or as tissue-like aggregates. 3D scaffolds have been generated from purified molecules such as collagen I, synthetic biomaterials, and even from native ECM from which living cells were previously extracted [304]. There are two mostly used methods. Epithelial cells can be completely embedded within the ECM where cells are grown in a gelled bed in the presence of culture medium containing growth factors and hormones that are necessary for proliferation and survival. In the overlay method, the ECM is first cast to form a gelled bed measuring 1 mm in thickness. Epithelial cells are seeded onto this bed as a single-cell suspension in culture media. Other less used methods include gyratory rotation and spinner flask spheroid cultures, microcarrier beads and pre-fabricated engineered scaffolds [179].

3D cultures have several important advantages over 2D cultures (Fig. 1.2). Cellular heterogeneity: 3D cultures are composed of cells with different phenotypes caused by mass transport limitations, such as proliferating, non-proliferating and necrotic cells, very similar to the situation within intact human tumors. It is far more realistic than the cellular homogeneity found in monolayer culture [159]. Matrix stiffness: the stiffness (compliance) of the ECM regulates multiple cellular functions [78]. Cancer cells sense external pressure through bidirectional interaction between cells and the surrounding ECM and respond accordingly. It has been reported that cell surface integrin receptors and the contractile cytoskeleton pull against the ECM to sense the stiffness of the microenvironment [109]. Cells respond to different stiffness of matrix by shifting distribution of cell surface integrin receptors and the types of cell adhesions and cytoskeletal structures formed, altering distribution of cell surface integrin receptors and the types of cell adhesions and cytoskeletal structures formed, and enhancing cell proliferation to promote neoplasia [62, 221]. That explains that pathological processes such as fibrosis or microenvironmental alteration within and around developing tumors can alter cell behavior due to the change in tissue stiffness and cellular responses [89]. For example, dense, non-pliable desmoplastic tissue is associated with some carcinomas [221] and sites predisposed for secondary metastases [154]. Cell and tissue polarity is a common property in epithelial cells. These cells have the apical and basal surfaces crucial for tissue organization and directional secretion of products. Their basal surfaces rest on thin, flat basement membranes comprised of collagen IV, laminin, and many other matrix proteins [304]. In normal breast tissue, epithelial cells are organized into spherical

3D structures surrounding a lumen to function as acini of glands. While cell polarity is preserved by culturing cells on a layer of collagen with other stromal factors, this organization is lost when these cells are explanted onto flat 2D tissue culture substrates [118]. Metastasis: the mechanisms of cell invasion in cancer have been well analyzed in 3D model systems. Local cleavage of the surrounding matrix by transmembrane proteases of the membrane
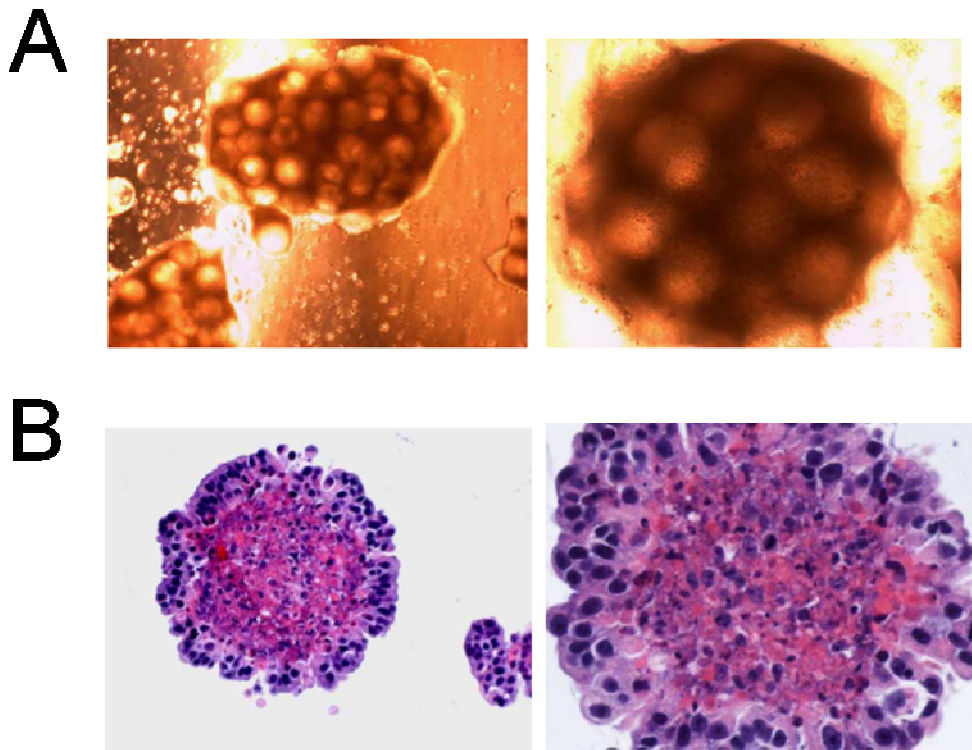
Fig. 1.2   Three dimensional culture of breast cancer cells

(A) Brightfield images of heterologous tumor–stromal spheroids (containing MCF-7 tumor cell line, human mammary fibroblasts and endothelial cells) (taken at ×75 and ×150 magnification). (B) H and E sections of heterologous tumor–stromal spheroids (containing MCF-7 tumor cell line, immortalized human mammary fibroblasts and endothelial cells)(taken at ×150 and ×300 magnification) [159].

type matrix metalloproteinases (MT-MMP) family is required for cell proliferation in model tumors and integrin-mediated invasion into collagen gels [134]. Similarly, MT1-MMP allows the expansion of normal endothelial cells to form new blood vessels and promotes the differentiation of pre-adipocytes into white adipocyte tissue. In addition, tumor cell adherence and migration in spheroid cultures can reflect the distinct metastatic potential of breast tumor cells [52]. Cell signals: human 3D *in vitro* epithelial models of mammary acini can mimic the increasingly abnormal tissue organization characteristic of breast carcinoma progression, where tumor cells suppress normal apoptotic mechanisms to invade the lumen [72]. Oncogene HER2 is reported to interact with the Par polarity complex components partition protein 6 (Par6) and atypical protein kinase C (aPKC) [11]. Inhibition of β1-integrin, EGF receptor, MAPK, PI3K, or Par6 can restore acinar architecture to a state closer to normal [11, 187]. Furthermore, analyzing patterns of gene expression in 3D *in vitro* mammary acini may be useful for predicting breast cancer outcome [35]. Genetic phenotype: many cells types, present in 3D models, were found to assume a near normal cellular architecture and exhibit gene expression profiles that were reflective of an authentic differentiated phenotype [264]. 3D *in vitro* culture systems have been shown to recapitulate the drug sensitivity patterns of tumor cells *in vivo*. 3D multi-cellular tumor spheroids from the breast cancer cell line, MDA-MB-231, exhibited a much lower IC_{50} to cisplatin when plated in 2D monolayer cultures than as suspended spheroids. Treatment of MDA-MB-231 spheroids, but not MDA-MB-231 monolayers, by cisplatin demonstrated up-regulation of TGF-ß mRNA and protein which is highly predictive of the patterns of drug response of tumor cells *in vivo* [217].

HER2/Neu

Human epidermal growth factor receptor 2 (HER2) and the rat homologue, Neu, belong to ERBB/HER family named from their homology to the erythroblastoma viral gene product, *v-erbB*. This receptor tyrosine kinase (RTK) family includes four receptors HER1–4 and 13 polypeptide extracellular ligands [54] (see Fig. 1.3). An important defining feature of the HER network is that two members of the family, HER2/Neu and HER3, are non-autonomous [54]. HER2 lacks the capacity to interact with a growth-factor ligand, whereas the kinase activity of HER3 is defective [123, 163]. Both HER2 and HER3 form heterodimeric complexes with other ERBB receptors that are capable of generating potent cellular signals.

HER2 plays a critical role in human development. In human fetuses, HER2 has been detected in heart, the nervous system, developing bone, muscle, skin, lungs and intestinal epithelium [60]. For instance, HER2 and HER4 are expressed in the myocardium, the underlying muscular portion of the atrium and the ventricle [54]. Signals from neuregulin-1 to the HER2/HER4-expressing myocardium initiates ventricular differentiation. A study shows HER2-mutant mice are embryonic-lethal as the lack of HER2 signaling undermines trabeculation in heart development, and the mutant heart is characterized by an irregular beat [180]. Normal breast growth and development is another example of HER2 involvement after birth. The EGF-like ligands and neuregulins that bind to this receptor family have been shown to stimulate the lobulo-alveolar development of the mouse mammary gland in explant cultures and *in vivo* [76].

The HER family of receptors are located at the cell membrane and share a similar structure, comprising a cysteine-rich extracellular growth factor (ligand) binding domain, a lipophilic transmembrane segment and an intracellular tyrosine kinase domain with a regulatory carboxyl-terminal segment [283]. Ligands activate HER receptors via receptor dimerization which results in transphosphorylation in their intracellular tyrosine kinases, providing a dock with numerous intracellular signaling molecules leading to activation of downstream second messenger pathways and cross talk with other membrane signaling pathways [15, 54]. Although no direct ligand has been identified, HER2 can be activated via heterodimerization with another family member or homodimerization with itself when expressed at very high levels [122]. In fact, HER2 is a preferred heterodimeric partner of the other three HER members. HER2-containing heterodimers have a higher affinity and broader spectrum of ligands than other heterodimeric receptor complexes [122]. Also, HER2-containing heterodimers are marked by slow endocytosis and frequent circulation back to the cell surface [17, 181, 297]. In sum, these features translate to more potent mitogenic signals [228].

Amplification of the HER2 gene (generation of more than the normal two gene copies) is the most common mechanism leading to increased HER2 protein expression, disruption of normal control mechanisms and formation of aggressive tumor cells [140, 143]. Amongst the most prominent pathways are the PI3K/AKT and the MAPK pathway.

HER2 induces transformation through PI3K/AKT pathway in cooperation with HER3 [8, 133]. A study shows HER3 provides HER2 the p85 binding motifs to PI3K [231, 248, 260]. This is further supported by tumors from MMTV-*neu* mice and HER2 overexpressing human breast tumor have activation of PI3K signaling [9, 274, 315]. The activation of PI3K/AKT regulates numerous cellular functions in cancer cells including cell proliferation and survival, cell size and response to nutrient availability, glucose metabolism, epithelial-mesenchymal transition and cell invasiveness, genome stability, and angiogenesis [191, 220, 271, 284].

The transforming functions of HER2 could also be mediated through Src kinase. Evidences suggest there is an association between Src activation and HER2 overexpression in MMTV-*neu* transgenic mice and HER2-positive breast carcinomas [295]. It has been reported that HER2 activates Src through increasing its expression and stability, or by directly phosphorylating Src on Tyr215 in its SH2 domain [269, 280]. Given that Src regulates focal adhesions and integrin signaling, and regulation of the actin cytoskeleton, this activation in Src will result in an up-regulation in cell migration, invasion, and metastasis [295]. In addition, as a downstream signal, Src may play a role in upstream activation. One study indicated c-Src enhances HER2-HER3 dimerization and increases their phosphorylation and signaling activities [146]. c-Src also phosphorylates HER2 at Tyr877 within the activation loop of the kinase domain and increases the kinase activity of HER2 [301].

HER2 signaling can disrupt cell polarity and cell adhesion. HER2 receptors are normally expressed on the basolateral surface of epithelial cells where it likely mediates cross talk with ligand secreting stromal cells [37, 69, 254]. The activation of HER2 leads to disruption of tight junctions, loss of cell polarity, and proliferative disarray in breast epithelial cell acinar structures [208]. This is partially mediated through its interaction with components of the Par polarity complex including PAR6 and aPKC [11]. On the other hand, the heterodimerization of HER2 with HER1 promotes the invasive phenotype mediated through pathways including PI3K, RAS, PLCγ, STAT3, PKC-α, FAK [23, 145, 313]. A few important studies reported that HER2 physically interacts with β4 integrin [90] and mammary tumors in MMTV-*neu* mice have delayed onset and reduced invasion and metastases if β4 integrin signaling is disrupted genetically [121].

HER2-positive breast cancer

The HER2 gene is amplified in approximately 20 to 30% of breast cancers patients. Breast cancers can have up to 25–50 copies of the HER2 gene, and up to 40–100 fold increase in HER2 protein resulting in 2 million receptors expressed at the tumor cell surface [151]. Half of HER2-positive breast cancers are ER-positive but they generally have lower ER levels, and many have p53 alterations [277]. HER2-positive breast cancer is associated with aggressive tumor behavior characterized by significantly shorter overall survival rate and time to relapse [143].

Two drugs are currently FDA approved for treatment of HER2 positive cancers. Trastuzumab is a humanized monoclonal antibody that recognizes the external domain of

HER2. HER2-positive breast cancer patients respond well to sole trastuzumab treatment [285]. Also, trastuzumab has a synergistic effect when combined with a variety of chemotherapy drugs. For instance, in clinical trials, trastuzumab increased the objective response rate and time to breast tumor progression when combined with doxorubicin, cyclophosphamide, or paclitaxel [138]. In addition, trastuzumab is beneficial when combined with endocrine therapy in both ER and HER2 positive patients [156]. Lapatinib is a small molecule tyrosine kinase inhibitor that blocks the kinase activity of HER1 and HER2 [204]. It is commonly used as a treatment in ER+/EGFR+/HER2+ breast cancer patients (now often called "triple positive") and in patients who have HER2-positive advanced breast cancer that has progressed after previous treatment with other chemotherapeutic agents, such as anthracycline, taxane-derived drugs, or trastuzumab. A 2006 GSK-supported randomized clinical trial on female breast cancer patients previously being treated with those agents (anthracycline, a taxane and trastuzumab) demonstrated that administrating lapatinib in combination with capecitabine delayed the time of further cancer growth compared to a regime that uses capecitabine alone. The study also reported that risk of disease progression was reduced by 51%, and that the combination therapy was not associated with increases in toxic side effects [108]. The outcome of this study resulted in a somewhat complex and rather specific initial indication for lapatinib—use only in combination with capecitabine for HER2-positive breast cancer in women whose cancer have progressed following previous chemotherapy with anthracycline, taxanes and trastuzumab.

The synergistic effect of lapatinib with trastuzumab is well documented. While trastuzumab fails to bind to the p95 truncated variant of HER2 (p95 HER2), lapatinib remains strong affinity to p95 HER2 and inhibits cell proliferation in trastuzumab-resistant cells expressing p95 HER2 [245]. It may also circumvent trastuzumab resistance associated with up-regulation of IGF-1R signaling [102]. In addition, lapatinib retains anti-tumor activity in PTEN-null HER2-overexpressing cell lines whereas trastuzumab does not [299]. In clinical study, this drug has been shown to cause remissions in trastuzumab-resistant patients and it may be more effective when given together with trastuzumab [36]. Also, the combination of lapatinib with certain chemotherapy drugs in patients with metastatic disease, has shown promising outcomes compared to the chemotherapy drug alone [108].
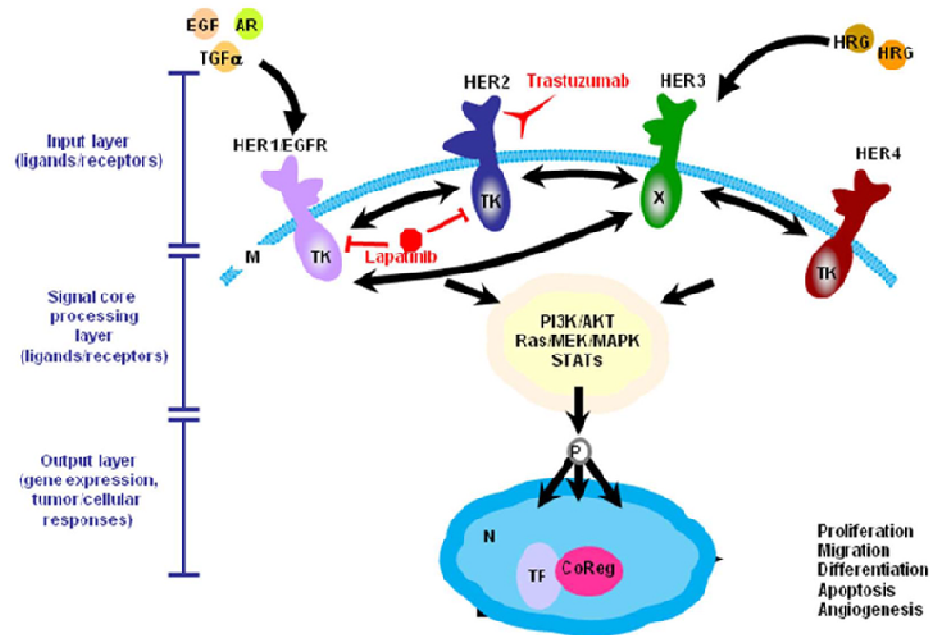
Fig. 1.3   The HER signaling network and HER2-targeted therapy in breast cancer

The HER network is a network comprised of an input layer of 4 membrane (M) tyrosine kinase (TK) receptors (HER1/EGFR-HER4) and multiple ligands [e.g., EGF, TGFα, and heregulins (HRG)]; a signal core processing layer involving a series of phosphorylation (e.g., activation of the PI3K/AKT, RAS/MEK/MAPK, and STATs kinase cascades) that transmit signals from the receptor layer to the output layer to alter expression of genes regulating tumor cell proliferation, survival, and other characteristics of the malignant phenotype. HER2 does not have a ligand, but exists in an open conformation exposing its dimerization domain; it can be activated by hetrodimerization with other ligand-bound HER members or by homodimerization when it is overexpressed. HER3 lacks the TK activity (X). Trastuzumab is FDA-approved in both the metastatic and the adjuvant settings and the dual HER1–HER2 small molecule TK inhibitor lapatinib is FDA-approved in metastatic HER2+ breast cancers [122].

Prolactin

Prolactin is a polypeptide hormone that is synthesized in and secreted from specialized cells of the anterior pituitary gland, the lactotrophs. This hormone was given its name based on the fact that an extract of bovine pituitary gland would cause growth of the crop sac and stimulate the elaboration of crop milk in pigeons or promote lactation in rabbits. Based on its genetic, structural, binding and functional properties, PRL belongs to the PRL/growth hormone (GH)/placental lactogen (PL) family group I of the helix bundle protein hormones [98].

The structure of human PRL is similar to growth hormone. It is an amino acid chain folded by three intra-molecular disulfide bonds between six cysteine residues (Cys^4-Cys^{11}, Cys^{58}-Cys^{174}, and Cys^{191}-Cys^{199} in humans) [58]. The sequence homology is around 56% between primates and rodents [257]. In rats [59] and mice [167], pituitary PRL consists of 197 amino acids, whereas in sheep [184], pigs [183], cattle [287], and humans [256] it consists of 199 amino acids with a molecular mass of ~23,000 Da.

PRL is a very versatile hormone. It plays roles in lactation, luteal function, reproductive behavior, immune response, osmoregulation, and angiogenesis. And certainly, its cancer-promoting effect will be discussed at the end of this chapter.

Location

PRL is found to be produced in various locations in the human body. A group of cells in anterior pituitary gland was first described to synthesize and secret PRL in light

microscopy [132]. These heterogeneously shaped cells [68] are sparsely distributed in the lateroventral portion of the anterior lobe and are present as a band adjacent to the intermediate lobe [211]. They are named as lactotrophs.

PRL was also detected in brain first by Fuxe *et al.* [101]. PRL immunoreactivity was found in the telencephalon in the cerebral cortex, hippocampus, amygdala, septum [74], caudate putamen [88], brain stem [75], cerebellum [253], spinal cord [131], choroid plexi, and the circumventricular organs [272].

Also, female reproductive organs such as placenta, amnion, decidua, and uterus have also been proved to produce PRL-like hormones. Among them are a family of placental lactogens found in the rat, mouse [255], hamster, cow [10], pig [95], and human [129]. Recent data showed PRL can be produced in the male reproductive organ such as prostate gland [107].

Lymphocytes are another source of PRL. Studies indicated immune-competent cells from thymus and spleen as well as peripheral lymphocytes contain PRL mRNA and release a bioactive PRL that is similar to pituitary PRL [77].

In fact, although a large amount of PRL in milk originates from the pituitary gland [120], the epithelial cells of the lactating mammary gland [213] are thought to self-synthesize PRL as well. PRL mRNA [213] as well as synthesis of immunoreactive PRL has been detected by mammary epithelial cells of lactating rats [189]. In addition, the mammary gland may also act as a posttranslational processing site for PRL given the fact

that the number of PRL variants in both human milk, far exceeds that found in serum [86].

Regulation

Pituitary PRL is regulated by dopamine secreted from the midbrain, substantia nigra pars compacta, and hypothalamus [98]. In lymphocytes and in decidual cells, PRL expression is stimulated by cAMP [107]. Lymphocytes also contain dopamine receptors that may be involved in the regulation of lymphocytic PRL production/release [73]. Progesterone up-regulates PRL synthesis in the endometrium but is a potent inhibitor in myometrium and breast glandular tissue [316].

During pregnancy, high circulating concentrations of estrogen and progesterone inhibit the action of PRL on milk production. Following delivery, reduced estrogen and progesterone production allows PRL to induce lactation. After childbirth, PRL levels fall as the internal stimulus is removed. Sucking by the baby on the nipple then promotes further PRL release, maintaining the ability to lactate [98].

Prolactin Receptor

The human PRL receptor (PRLR) belongs to class 1 of the cytokine receptor superfamily. The PRLR gene is located on chromosome 5 and contains at least 10 exons [18]. The PRLR exists as seven recognized isoforms as an result of transcription starting at different promoters or alternative splicing of the transcript [135] (Fig.1.4). They

include the long isoform, intermediate isoform, two short isoforms, ΔS1 isoform, PRLBP and TM-LCD among which the first four are of the most important in PRL signaling.

The long PRLR was the first human PRLR isoform identified with the longest sequence and is a classic type I single-pass cell-membrane receptor that consists of an extracellular domain (ECD), a transmembrane domain (TMD) and an intracellular domain (ICD). It is a polypeptide of 211 amino acids and is around 85 kDa. ECD contains two type III fibronectin-like domains, termed the S1 and S2 domains. The S1 domain contains the majority of ligand contact sites. The S2 domain has a smaller surface area for interacting with ligand but also contains elements responsible for interacting with its partner receptor in the ligand-dimerized complex. These structures contribute to the high affinity of the PRLR for PRL. The ICD contains Box 1, Variable Box (V-Box), Box 2, and Extended Box 2 (X-Box) motifs. The Box 1 motif provides docking site for Janus kinase 2 (JAK2). The function of the C-terminal region of the ICD is thought to bind to the signal transducer and activator of transcription 5 (STAT5) and SH2-containing protein tyrosine phosphatase (SHP-2) [7].

The intermediate PRLR isoform (50kDa) is truncated in its C-terminus. This results in a deletion of all coding sequence from C terminal to the X-Box. This isoform still engages JAKs but is incapable to activate Fyn tyrosine kinase. The intermediate isoform was unable to trigger the proliferation of transfected cells in response to ligand [164].

The S1a (56 kDa) isoform contains both the Box 1 and 2 motifs, whereas the S1b (42kDa) PRLR contains only the Box 1 element. Both short isoforms appear inert from a signaling perspective and may serve as ligand traps that function to either internalize ligand and/or down-regulate PRL-induced signaling [98].

ΔS1 (70kDa) isoform is missing the entire S1 domain. The affinity of the ΔS1 homodimer for ligand is reduced by approximately 7-fold. Interestingly, the dose-dependent activation of associated signaling cascades after ligand stimulation is only modestly delayed [165].

Human Prolactin Receptor Isoforms
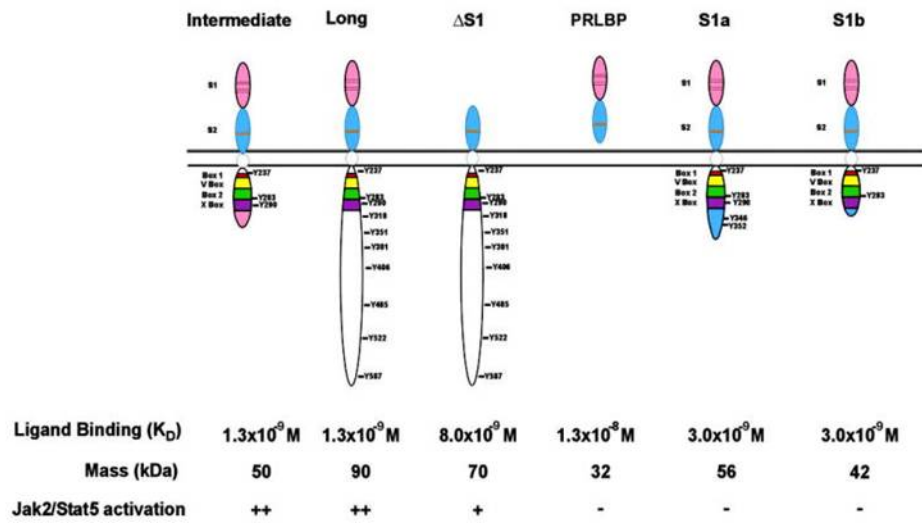
Fig. 1.4   Schema of the seven known human prolactin receptor (PRLR) isoforms

Extracellular and intracellular domains are indicated by 'ECD' and 'ICD', respectively, and the single transmembrane domain is represented by the small circle within the membrane.   Designations within the ICD represent the known tyrosine residues in the PRLR, and the colored boxes represent the Box 1, V-Box, Box 2 and X-box (left to right) [56].

Prolactin in Breast Cancer

Epidemiology

Hankinson *et al.* conducted a large prospective nested case-control study of plasma PRL levels with premenopausal breast cancer risk (*n*=235 cases). They observed a significant positive association, with a RR comparing the top versus bottom quartiles of PRL levels of 1.5 (95% CI=1.0–2.5) [279]. This association was strongest for ER+/PR+ tumors (RR=1.9, 95% CI=1.0–3.7) and for women over age 45 years (RR=2.3, 95% CI=1.1–5.0). In the other two large studies of premenopausal and postmenopausal women, PRL tended to be strongly associated with risk of breast cancer among women who were diagnosed within 4 years of their blood collection. However, in postmenopausal women, a statistically significant positive association between PRL and breast cancer risk was still observed among cases diagnosed 4–10 years after blood collection [279].

Other studies have reported that positive staining for PRL in about 80% of malignant breast tumors [31]. Increased PRL positivity was significantly associated with increased tumor size, higher stage, nodal involvement, and a worse overall survival [30]. In addition, high pretreatment PRL concentrations have been associated with treatment failure for both tamoxifen and aromatase inhibitors in most studies [16, 29]. Finally, prolactinomas, a condition characterized by extremely high PRL levels, may be associated with breast cancer development.

Prolactin and tumorigenesis

In breast cancer, PRL prevents apoptosis [176], enhances tumor cell proliferation, promotes angiogenesis, and increases cell motility and metastasis [115, 186, 194, 247]. PRL mediates its effects via PRLR activating the JAK2–STAT5 [267], RAS-RAF-MAPK, and PI3K-AKT pathways [4].

Both endocrine and autocrine/paracrine sources for PRL exist in mammals. In the 1980s, several clinical trials were conducted on breast cancer patients with pharmacological agents that inhibited the pituitary secretion of PRL aiming to inhibit tumor growth induced by PRL. The failure led to more thorough investigation in the extrapituitary source of PRL [55]. More and more evidences support the fact that the synthesis of PRL can locally occur in breast epithelial cells. A study indicated that hypophysectomized breast cancer patients had near-normal PRL levels [172], whereas immunohistochemistry studies revealed the expression of immunoreactive PRL protein in human breast epithelium [213]. Others suggest that the mRNA for PRL could be found in normal and neoplastic human breast epithelium and mammary epithelium from pregnant rodents [94]. In fact, 98% of human breast cancers synthesize PRL mRNA as detected by *in situ* hybridization [237]. Other than mammary gland, PRL was also found locally expressed in uterus and immune system [55]. Incubation of myometrial or leiomyoma cells with anti-PRL antibodies causes a significant decrease in cell number, supporting a role for PRL as a paracrine/autocrine growth factor [214]. PRL has also been detected in preterm cervical mucus [215] and in about 50% of uterine cervical

carcinomas [192]. A B-lymphoblastoid cell line, IM-9-P, produces relatively high levels of PRL [105]. A myeloid leukemic cell line and myeloblasts from patients with acute leukemia produce PRL [168], as do several non-Hodgkin's lymphoma cell lines [193].

While PRL significantly contributes to tumorigenesis, PRLR plays a crucial role as well. The studies examining PRLR expression at the mRNA level have suggested an association with either ER/PR expression [219] or neoplasia [198]. The expression of the PRLR occurred in 80% of human breast cancers, generally in association with the expression of estrogen receptor/progesterone receptor (ER/PR) [198].

PRL activates a member of the JAK family, primarily JAK2, upon PRLR dimerization. This provides docking sites for proteins with SH2 domains, including STATs. As previously discussed, the interaction of JAK2 with the PRLR appears to be mediated by an interaction of the membrane-proximal Box 1/Box 2 motif of the PRLR with the N-terminus of JAK2 [96, 178]. Loss of JAK2 activity results in ablation of PRL-induced STAT5 phosphorylation and downstream gene expression [40]. JAK2 activity is necessary for the *in vitro* [300] and *in vivo* [241] growth and differentiation of mammary cells and tissues.

STAT5 is the downstream signal activated by JAK2. In commonly studied mammary tumor cell lines, including T-47D, MCF-7, and BT-20, PRL treatment results in increased tyrosine phosphorylation of STATs 1, 3, and 5 [66, 190]. Several studies have demonstrated increased levels of STATs 1 and 3 in primary mammary tumors [38], and the incidence of elevated STAT5 activation in other tumor types [38] suggests a high

probability that these STATs may be elevated in mammary tumors as well. Kazansky and Rosen have demonstrated that STAT5b, but not STAT5a, is a potent mediator of Src-induced tumorigenesis [157]. STATs are altered by multiple hormones, growth factors, and signaling cascades, pointing to an obvious role they may play in cross talk with many other agents important in mammary carcinogenesis. Activation of the STAT complex engages its DNA binding sequence, resulting in promoter transactivation under appropriate conditions [188].

A second pathway that has received focused attention in mammary tumor cells is the RAS-RAF-MAPK pathway. PRL has been shown to activate this pathway in a number of PRL-dependent models and mammary tumor cell lines, as well as normal mouse mammary epithelial cells [57]. In T-47D cells, this was associated with increased association of SHC with JAK2, as well as GRB2 and SOS, indicating a role for JAK2 in this cascade. The p42/44 MAPKs are linked to proliferation for many growth factors in many systems and also appear to be linked to PRL-induced proliferation of mammary tumor cells [64-65]. In PRL-deficient MCF-7 cells, a MEK1 inhibitor decreased proliferation of unstimulated cells. EGF, but not PRL, was able to overcome this inhibition, indicating a critical role for this pathway in PRL, but not EGF-stimulated proliferation [247]. PRL also can synergistically activate this pathway, via cross talk with other growth factors, depending on the phenotype of the tumor cell. PRL-induced activation of JAK2 resulted in tyrosine phosphorylation of HER2, thereby increasing association with GRB2, and activating the RAS-MAPK pathway [306]. p42/44 MAPKs are believed to exert these effects on proliferation via multiple mechanisms, including

phosphorylation of Ets transcription factors, increasing synthesis of the *fos* gene family (c-*fos*, Fra-1,2, c-*jun*, JunB), phosphorylation of carbamoyl phosphate synthetase II, leading to increased DNA synthesis, as well as many other protein kinases and other substrates in the cytoplasm, indirectly modulating downstream activity [55].

PI3K is another pathway involved in PRLR activation. p85, the regulatory subunit of PI3K becomes associated with the PRLR after ligand exposure in transfected human embryonic kidney 293 and Chinese hamster ovary cells [25, 305]. PRLR association with Src family members contributed to PI3K activation in Nb2 cells [5]. PI3K could potentially be activated by PRL through multiple additional pathways. It can be a target of RAS [239], and the p85 regulatory subunit has been shown to associate with several downstream effectors and adaptors of cytokine and growth factor receptors, including STAT5, STAT3, IRS 1, Gab1 and Gab 2, and SHP-2 [225, 242], all of which have been shown to be activated by PRL, or are associated with the activated PRLR in some way . PI3K-generated phosphoinositides provide docking sites for AKT (protein kinase B), which activate AKT by threonine/serine phosphorylation. This pathway initiates survival, inhibits pro-apoptotic signals [153, 169], and also modulates regulators of cell cycle progression such as E2-F, and cyclin D1 [110, 206]. Indeed, expression of activated AKT retarded mammary involution and contributed to mammary tumor progression *in vivo* [141, 249].

Prolactin Receptor Cross Talk

Mammary development requires coordinated interactions of multiple growth factors and hormones. As mentioned earlier, the downstream signaling pathways of PRLR are shared by many of these factors and represent potential sites for cross talk, as well as putative sites for therapeutic intervention [44].

Accumulating evidence points to synergistic interactions between PRL and estrogen, members of the EGF family, and IGFs. These factors are the targets of endocrine and molecular therapies, and continue to be the focus of pharmaceutical development. However, acquired resistance to existing therapies after the initial patient response has proven a major obstacle in clinical oncology [44]. With the abundance of evidence implicating PRL in the cross talk with these proliferative factors in breast cancer, PRL and/or PRLR become promising targets.

PRL cross talk with HER2 and EGFR

A number of effects of phosphorylation dependent cross talk have been noted for PRL and EGF. Some of these effects may depend on the cellular context (normal vs. malignant) in which the cross talk occurs. Sheffield and colleagues conducted an elegant series of studies using the normal murine mammary epithelial cells (NMuMG) model system to examine the effects of PRL on EGFR function [91-92, 149, 234]. In those studies, PRL caused activation of PKC, but only modest MAPK activation and PRL treatment resulted in EGFR threonine phosphorylation. This PRL-induced EGFR phosphorylation was inhibited by a specific PKC inhibitor. In the NMuMG cells, PRL

decreased both basal and EGF-induced EGFR tyrosine kinase activity. Notably, this PRL-induced desensitization of the EGFR was reversed by in vitro dephosphorylation of the isolated receptor by treatment with alkaline phosphatase, suggesting it was accounted for by the increased threonine phosphorylation. Furthermore, PRL co-treatment dramatically inhibited EGF-induced RAS/MAPK signaling and EGF-induced mitogenesis. Thus, in NMuMG cells, PRL supressed EGFR function by causing PKC-dependent threonine phosphorylation of EGFR. In contrast to this inhibitory effect of PRL on EGF signaling in normal mammary epithelial cells, other authors studying human mammary carcinoma cells have observed crosstalk between PRL and EGF with regard to cell migration and gene activation [56, 130, 194]. For example, the Clevenger laboratory demonstrated that several human breast cancer cell lines including T-47D, MCF-7 and MDA-MB-231 exhibited synergistically increased motility when treated with the combination of PRL and EGF compared to the responses to each individual factor [194]. This differential response in normal vs. cancerous mammary cells suggests potentially important context-specific elements of cross talk between these two factors. Frank *et al*. examined further PRL-EGF cross talk and the effects of PRL on EGFR and HER2 in T-47D cancer cells [136]. They found that both PRL and EGF caused robust signaling in T-47D cells. PRL activated JAK2, STAT5, and MAPK, while EGF caused EGFR activation and consequent SHC/MAPK activation. PRL caused phosphorylation of both EGFR and HER2 detected by MAPK inhibitors, revealing that this PRL-induced phosphorylation was dependent on the MAPK pathway, but not the PKC pathway. The addition of PRL to EGF treatment significantly retarded EGF-induced EGFR down-

regulation in an MAPK activation dependent manner and with a time course that correlated with the synergistic effects of PRL on EGF signaling. These data are notable in that they indicate that PRL synergistically augments EGF signaling in T-47D breast cancer cells at least in part by lessening EGF-induced EGFR down-regulation and that this effect requires PRL-induced MAPK activity and threonine phosphorylation of EGFR. Positive cross talk between PRL and TGF-α in murine mammary epithelial cells *in vivo* to MAPK and reduction of tumor latency is also observed in mouse models.

Cross talk with estrogen

Studies have shown that the ability of the rat corpus luteum to respond to estrogen requires PRL, which can stimulate the expression of the ER. Transcription of the genes encoding both ERα and ERβ is stimulated by PRL through the JAK2–STAT5 pathway and STAT5-response elements that are located in each of the *Esr* promoters. A single nucleotide difference between these two response elements is responsible for the observation that either STAT5a or STAT5b can stimulate *Esr1* transcription, whereas only STAT5b can activate transcription of *Esr2*. The tyrosine kinase JAK2 is required for PRL activation of *Esr1* promoter activity; however, additional pathways are involved in PRL-induced STAT5b phosphorylation, nuclear translocation and DNA binding. In addition to the corpus luteum, PRL-induced ER expression might provide a mechanism for the responsiveness of other target tissues, such as the decidua and mammary gland, to these two hormones [97].

Cross talk with IGF-II

Insulin-like growth factor (IGF)-II is a required for PRL-induced up-regulation of cyclin D1 and proliferation in normal murine mammary epithelial cells *in vivo* and *in vitro*. PRL up-regulated transcript levels of both IGF-I and IGF-II. Moreover, PRL increased cyclin D1 in the presence of the IGF-I receptor neutralizing antibody. On co-treatment, IGF-I and PRL elicited cooperative phosphorylation of MAPK and AKT, but not STAT5. This interaction extended to increased activation of activating protein-1 enhancer elements, phosphorylation of glycogen synthase kinase 3 β, induction of cyclin D1, and ultimately, increased cell number. It also increased invasive behavior, which correlated with elevated matrix metalloproteinase-2 transcript levels. Together, these data indicate that strong cross talk between PRL and IGF-I augments biological processes associated with tumor progression, with implications for therapeutic strategies [45].

Prolactin Receptor Antagonists

As described above, data indicate that anti-PRL therapies for breast cancer, solely aimed at PRL from the pituitary only, were bound to be unsuccessful. As a consequence, recent investigations have utilized various strategies that seek to block PRL action both at the endocrine and autocrine/paracrine levels. One approach is to develop PRL analogs that bind but do not trigger the PRLR activation, thereby functioning as PRLR antagonists.

Phosphorylated prolactin as a natural prolactin receptor antagonist

Under normal physiologic conditions, 30% or less of PRL is post-translationally modified via multiple mechanisms including proteolytic cleavage, glycosylation, and phosphorylation [257]. The primary amino acid phosphorylated in human PRL is a serine residue at position 179 [278]. In studies using the Nb2 proliferation assay, the accepted measure of PRL bioactivity, removal of the phosphorylation motif from rat PRL increased PRL-induced proliferation, demonstrating phosphorylation may inhibit the activity of this hormone [288]. Further studies demonstrated that small reductions in the levels of phosphorylated PRL (versus unphosphorylated PRL) led to increased overall biological activity suggesting that phosphorylated PRL may act as an antagonist to its unphosphorylated counterpart *in vivo* [288].

S179D-human PRL is a molecular mimic of phosphorylated PRL

The mutant peptide S179D-hPRL was first generated 1998 and resulted from the replacement of an aspartic acid in place of serine 179. It was assumed during the design of this mutant PRL that the negative charge of an aspartate residue can mimic a phosphorylated serine reside [50].

The Nb2 bioassay studies suggested that S179D might inhibit proliferation in a non-competitive manner as its antagonism could not be completely reversed by excess wild-type PRL [50]. Further studies have also revealed that S179D-hPRL can inhibit the growth of prostate cancer cell lines both *in vitro* and *in vivo* [298, 302].

In breast cancer cells engineered to be PRL deficient, S179D-hPRL was demonstrated to act both as a weak agonist and to antagonize unphosphorylated PRL activity, although the antagonism was incomplete [246]. These data have led to the suggestion that S179D-hPRL may be exerting its actions through an isotype variant of PRLR, termed the PRLR-S1b, instead of through the classical long isoform of the PRLR in prostate cancer cell line [298]. S179D-hPRL, perhaps acting through the PRLR-S1b, may be involved in inhibition of the PRL-mediated activation of STAT5, while activating MAPK [286].

G129R

The antagonist developed in our lab was initially designed based on the presumption of data from the crystal structure of the ligand-dimerized growth hormone receptor extracellular domain [70, 261]; given the similarities between the PRL and GH receptors, it was assumed that PRL induced the sequential dimerization of its receptor [51].

PRL has four α-helical segments that are connected via loops that provide flexibility and allow the helices to be bundled in an anti-parallel (up-up-down-down) manner. Two separate asymmetric receptor-binding regions have been identified in these hormones, each of which interacts with the equivalent region of a receptor to form a one-ligand, two-receptor complex. Due to differences in affinity between the two receptor-binding sites, binding occurs sequentially with the higher affinity site (site 1) interacting

50

with the first receptor before the lower affinity site (site 2) can interact with a second receptor [63, 100].

Studies of GH, PRL, and placental lactogen (PL) have shown that the third α-helix is important to their structure and the function of site 2. In our lab, substitution of a Gly residue in this region with a more bulky and charged Arg residue results in antagonist, G129R [51]. Thus, G129R binds a single PRLR but is impeded from binding a second receptor and forming active heterotrimeric complexes (see Fig.1.5). Our data reveal at modest concentrations, G129R binds receptors with a one to one stoichiometry, sequestering the receptor from functionally productive binding and activation by endogenous wild-type hPRL.

G129R was demonstrated to have an inhibitory effect on cell proliferation; an effect that was shown to synergize with the anti-estrogen agent 4-OH-tamoxifen in PRLR-positive T-47D breast cancer cells [51]. In addition, G129R was shown to up-regulate TGF-β1 (apoptotic factor) secretion and down-regulate TGF-α (survival factor) secretion in a dose-dependent manner in T-47D cells [235]. Further studies demonstrated that G129R activated caspase-3 to induce cell apoptosis [235] and down-regulated Bcl2 [20]. The pro-apoptotic effect of G129R was confirmed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay (TUNEL) among four PRLR-positive breast cancer cell lines tested [20]. G129R also inhibited the phosphorylation of STAT3, AKT and MAPK *in vitro* [47, 175]. *In vivo* studies in a murine tumor model showed that G129R inhibited T-47D and MCF-7 tumor growth, while PRL stimulated the *in vivo*

growth of the tumors [49]. In addition, transgenic mice expressing G129R under the control of the metallothionein promoter revealed a significant decrease in ductal branching and lobular bud formation in the mammary gland and interestingly tumor appearance was significantly delayed in these mice [51, 276]. Although it was demonstrated that G129R had significantly antagonized PRL/PRLR-mediated signaling cascades [20, 47, 51, 99, 190, 222], residual agonistic activity of these analogs was also observed in other cell bioassay systems [26, 99, 113] and in animal models [27, 200].
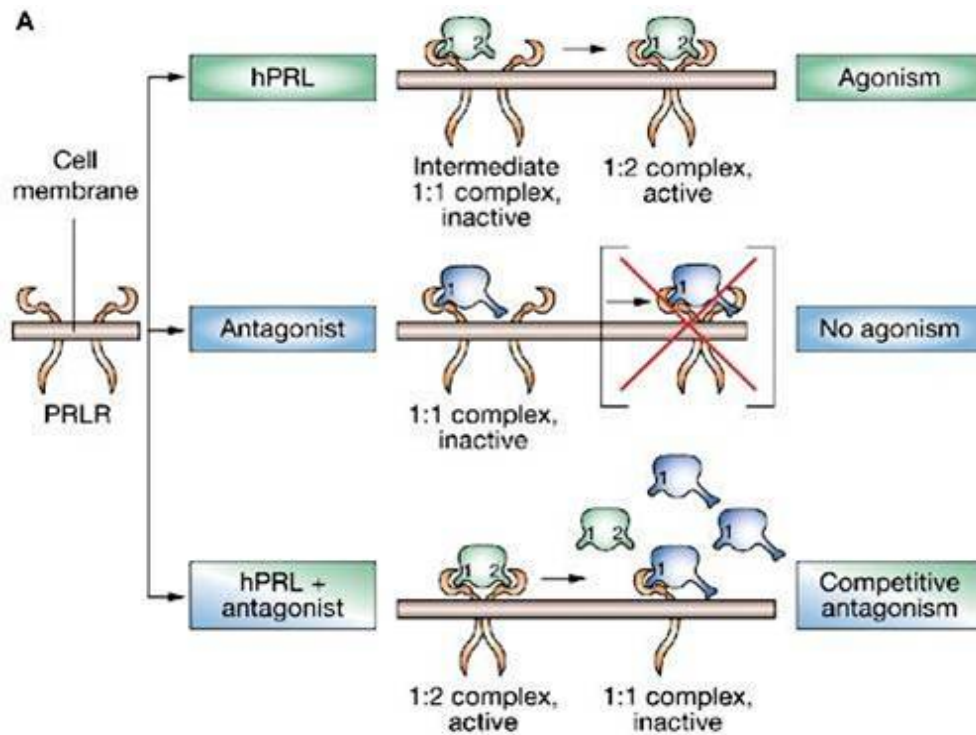
Fig. 1.5   Modeling the function and structure of prolactin-receptor antagonists

(A) Model of the interaction between PRL or PRLR antagonists with the PRLR. Since PRL is assumed to induce sequential receptor dimerization via interactions involving both binding sites (top panel), mutations introduced in binding site 2 of the antagonists are believed to prevent functional receptor dimerization (middle panel). When the antagonist is added in molar excess, it prevents PRL binding to the receptor, resulting in a lack of receptor activation and prevention of PRL actions (bottom panel) [114].

Δ1-9-G129R and Δ1-14-G129R

The Δ1-9-G129R and the Δ1-14-G129R [27] contain both the G129R replacement and deletion of the N-terminal nine or fourteen amino acids of PRL. The design of these mutants was based in part on crystal structure analysis of the placental lactogen/PRLR-ECD crystal structure, which revealed a critical role of the N-terminus of PRL in the interaction of site 2 with the PRLR [85]. Allegedly, the partial agonism observed with the G129R peptide was abolished in the Δ1-9-G129R and Δ1-14-G129R. Significant inhibition of PRL-induced cell proliferation and decreased levels of PRL-induced STAT5 phosphorylation were observed in Δ1–9-G129R or Δ1-14-G129R treated T-47D human breast cancer cells [27]. However, the antagonism was noted only when these peptides were used in at least 50-fold excess [27]. At the *in vivo* level, the Δ1-9-G129R was also found to block STAT5 activation in probasin-PRL transgenic mice, using doses of between 0.25 mg-1 mg/mouse day [27, 112]. In addition, diminished MAPK and STAT3/5 activation in PRL-stimulated murine mammary glands was noted following co-injection of a 50-fold excess of Δ1-9-G129R [27]. The affinity of the Δ1-9-G129R and the Δ1-14-G129R for the PRLR remains problematic.

G129R fusion proteins

G129R has a short half life *in vivo* [175]. To extend serum half-life (by increasing the molecular mass of the antagonist to inhibit its egress through the glomerular filtration apparatus) and add other potential anti-tumor functionalities, fusion

peptides between the G129R and potential anti-tumor peptides have been generated that include: G129R-IL2, G129R-endostatin, and, G129R-PE40-KDEL.

Interleukin-2 (IL-2) is a 15 kDa glycoprotein that stimulates the activation and proliferation of T lymphocytes and natural killer cells. Although IL-2 was one of the first cytokines used for tumor immune therapy in the generation of lymphocyte-activated killer (LAK) cells, its profound side effects currently limit its direct *in vivo* application. The G129R-IL-2 fusion protein was designed to treat breast cancer by combining PRL endocrine therapy and IL-2 immune therapy in one compound [314]. This fusion protein, G129R-IL2, was hypothesized to localize IL-2 in breast cancer tumor sites resulting in PRLR antagonism and activation of T lymphocytes for site-specific tumor cytotoxicity. When examined in vitro the G129R-IL-2 was demonstrated to inhibit PRL-induced STAT5 phosphorylation and breast cancer cell proliferation [314]. Treatment of Balb/c mice injected with EMT6 transfectants overexpressing PRLR with 100 µg G129R-IL-2/mouse per day, resulted in a statistically significant reduction in tumor size.

Endostatin is a recognized inhibitor of endothelial cell proliferation and angiogenesis [310], thus the fusion peptide was hypothesized to suppress PRL-induced tumor cell functions and reduce tumor-induced angiogenesis. The G129R-endostatin fusion protein was demonstrated to inhibit human umbilical vein endothelial cell (HUVEC) proliferation and the formation of endothelial tube structure *in vitro*. *In vivo*, nude mice injected with 4T1 cells demonstrated an enhanced half life in serum of the G129R-endostatin fusion protein and a statistically significant reduction of tumor volume

and mass in comparison to those mice that were treated with 5 mg G129R/kg/day or endostatin alone [19].

Another fusion peptide examined is the G129R-PE$_{40}$-KDEL construct. *Pseudomonas* exotoxin A (PE) is a bacterial toxin which inhibits protein synthesis via its PE$_{40}$-KDEL moiety. This fusion toxin competitively binds to PRLR on T-47D human breast cancer cells and inhibits STAT5 phosphorylation induced by hPRL. In addition, G129R-PE$_{40}$-KDEL is selectively cytotoxic to breast cancer cell lines expressing the PRLR and that cell death is associated with the inhibition of protein synthesis and does not involve caspase mediated apoptosis [174].

CHAPTER TWO

HYPOTHESIS AND OBJECTIVE

Previously, we reported that a PRLR antagonist, G129R, exerts an additive effect with humanized anti-HER2 monoclonal antibody, trastuzumab in female MMTV- *neu* transgenic mice [250], i.e. inhibiton of PRLR will enhance the inhibitory effect of trastuzumab on HER2. However, the effect of G129R on HER2 was abolished when it was used to treat primary tumor cells in 2D monoculture setting. I believe that the discrepancy of G129R's inhibitory effect on HER2 signaling *in vivo* and in tissue culture is, at least in part, due to the tumor microenvironment disruption in primary cell culture. Therefore, I hypothesize that the stromal-epithelial interactions play an important role in modulating the cross talk between PRLR and HER2/Neu in breast cancer.

My main objective in this dissertation study, therefore, is to investigate the mechanisms by which the tumor stroma exerts its influence on the cross talk between PRLR and HER2 in cancerous epithlium. Specifically, I plan to compare the response of tumor epithelium to G129R between epithelial monoculture and epithelial/stroma cells co-culture model. Furthermore, I will attempt to investigate the potential molecular mechanisms of epithelia-stroma interaction modulating PRLR and HER2 crosstalk using various systems such as coculture, transwell, and matrigel models. Finally, I plan to test the effect of G129R treatment in Neu activation in the *ex vivo* model and *in vivo* model.

CHAPTER THREE

MATERIALS AND METHODS

Transgenic Mice

Breeding pairs of FVB/N-Tg (MMTVneu) 202Mul/J mice, expressing the wild-type rat *neu* transgene under the control of the mouse mammary tumor promoter (MMTV-*neu*), were obtained from The Jackson Laboratory (Bar Harbor, ME). The colony was expanded and housed in accordance with *The Guide for the Care and Use of Laboratory Animals*. All animal studies were reviewed and approved by Clemson University Institutional Animal Care and Use Committee.

Preparation of Tumor Lysates

Tumors were resected (see Fig.3.1) with a sterile scalpel and washed in PBS, pH 7.4. Tissues were minced into paste with a sterile scalpel before being washed three times in PBS. The paste then was suspended in lysis buffer (50 mM Tris·HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA, pH 8.0; 1 μg/ml aprotinin; 1 μg/ml leupeptin; 1 μg/ml pepstatin A; 170 μg/ml PMSF; 180 μg/ml Na_3VO_4; 50 mM NaF) and homogenized using an electric homogenizer (250 mg tumor tissue per 1 mL lysis buffer). Lysates were harvested into microcentrifuge tubes, placed on ice for 15 min and centrifuged at 15,000 ·g for 15 min at 4ºC. Supernatants were collected and the protein content was determined using Coomassie Plus Protein Assay reagent and BSA standards (Thermo Scientific, Rockford, IL).
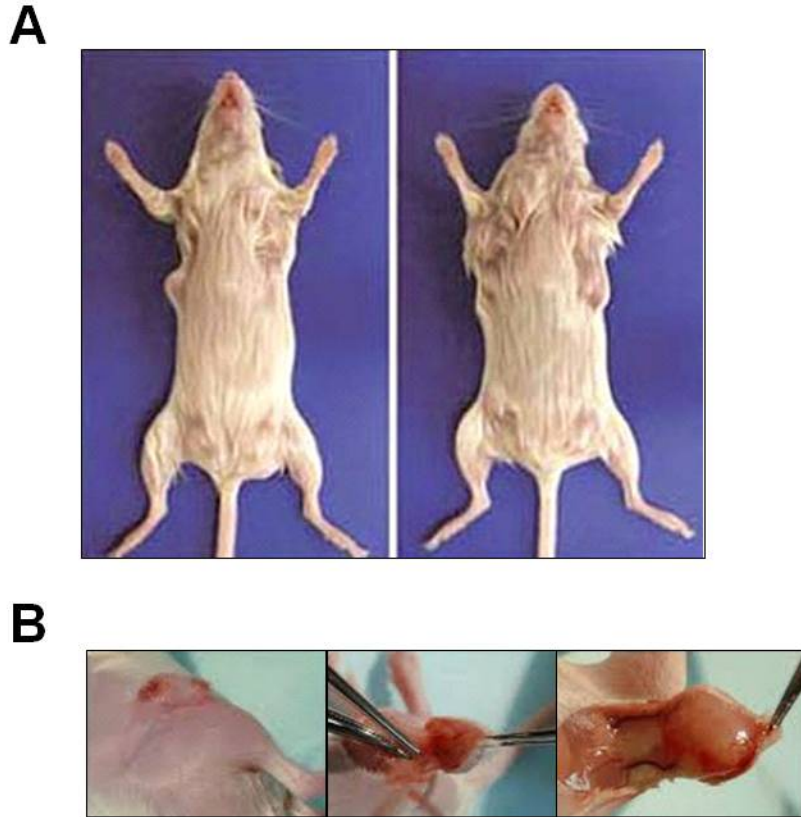
Fig. 3.1   Preparation of breast tumors in female MMTV- *neu* transgenic mice

Female MMTV-*neu* transgenic mice were killed with cervical vertebrate dislocation method (A). The breast tumors were identified and located. The surgical area was cleaned with 70% ethanol. (B) The surgical area was shaved and sterilized with 70% ethanol one more time. Breast tumors were resected by sterile scalpel or scissors [232].

Preparation of Lung Lysates

Whole lung tissues were resected with a sterile scalpel and washed in PBS, pH to 7.4. Lungs were carefully examined under dissecting microscope. Metastatic tumors in lungs were carefully resected using a sterile scalpel or scissors and tumors from an individual mouse were transferred together into a sterile petri dish. Lung tissues or tumors were minced into paste with a sterile scalpel before being washed three times in PBS. The paste was suspended in lysis buffer and homogenized using an electric homogenizer (250 mg tumor tissue per 1 mL lysis buffer). Lysates were harvested into microcentrifuge tubes, placed on ice for 15 min and centrifuged at 15,000g for 15 min at 4°C. Supernatants were collected and the protein content was determined using Coomassie Plus Protein Assay reagent.

Preparation of Mammary Gland Lysates

Five pairs of mammary glands were identified in the thoracic and abdominal region of the mice (see Fig. 3.2). Complete mammary glands were resected with a sterile scalpel and washed in PBS, pH 7.4. Tissues were minced into paste with a sterile scalpel before being washed three times in PBS. The paste then was suspended in lysis buffer and homogenized using an electric homogenizer (250 mg tumor tissue per 1 mL lysis buffer). Lysates were harvested into microcentrifuge tubes, placed on ice for 60 min and centrifuged at 15,000 ·g for 10 min at 4°C. The fatty layer on the top of the solution and the precipitate were removed. The clear middle layer of the supernatant
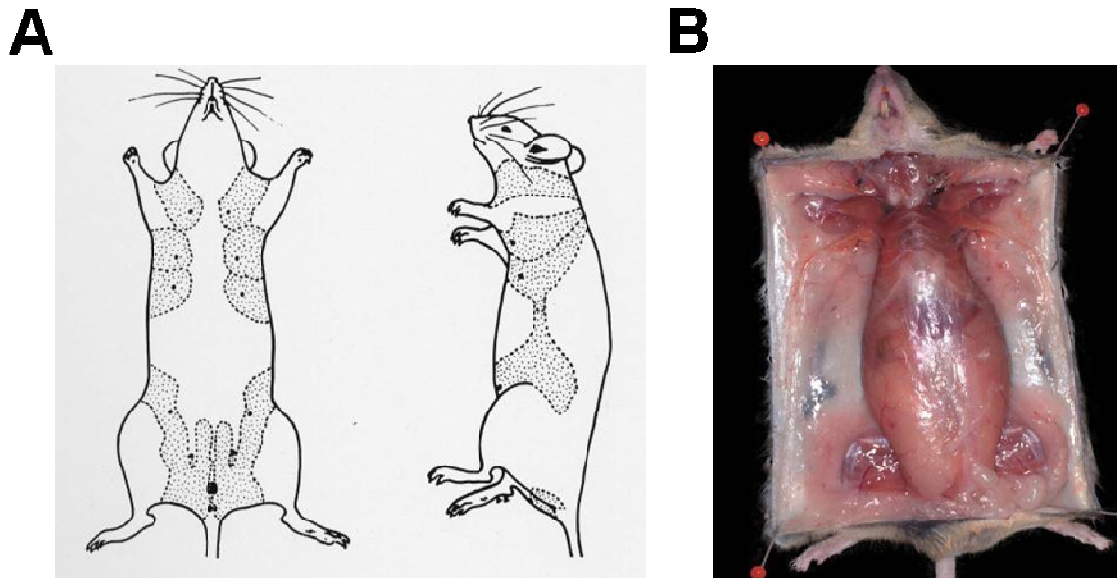
Fig.3.2   Preparation of mammary glands in female MMTV- neu transgenic mice

(A) Scheme reporting the localization of mammary glands (Murphy E.D., chapter 27 Characteristic Tumors, in E.L. Green Ed., "Biology of the Laboratory Mouse", reproduced by permission of McGraw-Hill, New York 1966). (B) A median longitudinal cut in mouse was made with a sterile scalpel and the skin was separated from the underlying musculature. The skin is then dissected and turned on one side and then on the other, so as to obtain an examination field as wide as possible. The characteristics of mammary glands and of the skeletal muscles will then be apparent [1].

was collected and the protein content was determined using Coomassie Plus Protein Assay reagent.

Coomassie Plus Protein Assay

Six standard solutions (1 mL each) containing 0, 250, 500, 1000, 1500 and 2000 µg/mL BSA were prepared. The Coomassie®Plus Reagent was allowed to warm up to room temperature (RT). The Coomassie® Plus Reagent solution was immediately mixed before use by gently inverting the bottle several times. 0.05 ml of each standard sample was pipetted into appropriately labeled test tubes. 1.5 ml of the Coomassie® Plus Reagent was added to each tube and mixed well. Samples were incubated for 10 minutes at room temperature (RT). With the spectrophotometer set to 595 nm, the instrument was calibrated on a cuvette filled only with reagent. Subsequently, the absorbance of all the samples was recorded. The average 595 nm reading for the Blank replicates was subtracted from the 595 nm readings of all other individual standard and unknown sample replicates. Finally, a standard curve was created by plotting the average Blank-corrected 595 nm reading for each BSA standard vs. its concentration in µg/ml. The standard curve was used to determine the protein concentration of each unknown sample.

For test samples, lysates were diluted 10 fold in distilled water before they were added into 1.5 ml of the Coomassie® Plus Reagent in each tube. Tubes were covered with parafilm and mixed well by inverting several times. Samples were subsequently incubated for 10 min at room temperature before being read in the spectrophotometer set

to 595 nm. Using the readings, protein concentration of lysates was determined according to the standard curve.

Cell Lines and Reagents

MCNeuA epithelial and N202Fb3 CAF clonal cell lines established from a female MMTV-*neu* mouse mammary tumor were kindly provided by Dr. Michael Campbell (University of California, San Francisco, CA) [43]. Human SK-BR-3 breast cancer cells and CRL-7236 primary human CAFs were obtained from the ATCC (Manassas, VA). Two types of primary mouse embryonic fibroblasts (MEFs) were derived from the embryos of non-transgenic (NTG-MEFs) and MMTV-*neu* transgenic (NEU-MEFs) FVB/N mice as described previously [209]. All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 10 µg/ml gentamicin at 37°C in a 5% CO_2 humidified atmosphere. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). The recombinant human PRL and its antagonist analog, G129R, used for all experiments were prepared in house as described previously [250].

Preparation of Tumor Chunks and Primary Tumor Cells

Spontaneous mammary tumors from female MMTV-*neu* mice were resected, rinsed in ice-cold phosphate buffered saline (PBS), and minced into tumor chucks (~3 mm^3) using a sterile scalpel. A portion of the tumor chunks were minced further, incubated in 0.25% trypsin-EDTA (Invitrogen) for 30 min at 37°C with constant mixing,

and filtered through a 100-µm cell strainer. The cell suspension was centrifuged at 800g for 10 min and resuspended and incubated in ACK Lysing Buffer (Lonza, Walkersville, MD) for 10 min prior to filtration through a 40-µm strainer. The single cell suspension of primary tumor cells were centrifuged at 300g for 10 min, resuspended in PBS, and the cells were counted with a hemocytometer.

FACs Analysis of Primary Tumor Cells

Primary tumor cells (3×10^5 cells) were suspended in 30 µl of rat anti-EpCAM (14-5791-81; eBioscience, San Diego, CA) diluted in PBS (1:1000), incubated for 15 min on ice, and centrifuged at 500g for 2 min. This process was repeated using goat anti-rat IgG-PE (sc-3740; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS (1:60), rabbit anti-PRLR(M170) (sc-30225; Santa Cruz Biotechnology) diluted in PBS (1:30), and goat anti-rabbit-IgG-FITC (sc-2012; Santa Cruz Biotechnology) diluted in PBS (1:60). Finally, the cells were suspended in 400 µl of PBS and the surface expression of EpCAM and PRLR were evaluated by flow cytometry with a FACSCalibur instrument using CellQuest software (Becton Dickinson, San Jose, CA). As negative controls, irrelevant antibodies of the same isotypes (Becton Dickenson) were used in replacement of anti-EpCAM and anti-PRLR. The fluorescence of PE and FITC was excited with an argon laser at 488 nm and detected at 570 nm and 530 nm, respectively.

Immunohistochemical and H&E staining

Tumor chunks were fixed with 10% neutral buffered formalin overnight, paraffin embedded, and sections (5 μm) were mounted on slides. Deparaffinized tissue slides were rehydrated, and heat induced epitope retrieval was performed in citrate buffer using a pressure cooker (20 min at 80 pKA). MCNeuA and N202Fb3 cells were mono- or co-cultured at a ratio of 4:1 on poly-prep slides (Sigma-Aldrich, St. Louis, MO) in DMEM supplemented with 10% FBS for 24 hrs at 37°C in a 5% CO_2 humidified atmosphere. Cells were fixed with 10% neutral buffered formalin for 10 min and permeabilized in 10% Triton X-100 for 10 min.

For immunohistochemical staining, endogenous peroxidase activity was blocked with 3% H_2O_2 in PBS for 10 min prior to blocking in horse serum for 30 min. Slides were incubated overnight in horse serum with a 1:2000 dilution of rabbit anti-cleaved caspase-3 (9961; Cell Signaling, Beverly, MA), a 1:500 dilution of rabbit anti-Ki-67 (ab16667; Abcam, Cambridge, MA), a 1:1000 dilution of mouse anti-vimentin (ab7752; Abcam), a 1:200 dilution of rabbit anti-E-cadherin (4065; Cell Signaling), or as a negative control a 1:200 dilution of mouse or rabbit IgG. The UltraVision ONE Detection System HRP Polymer and DAB Plus Chromogen kit (Thermo Scientific, Rockford, IL) was used to detect all antigens. The slides were counterstained with hematoxylin and bluing reagent and mounted with coverslips using Permount mounting medium (Fisher Scientific).

For H&E staining, deparaffinized slides were stained with hematoxylin for 5 min, washed with acetalcohol for 30 sec, and then stained with eosin for 2 min (all from Sigma). Slides were imaged with an Olympus microscope and images were obtained with a CCD cooled 1.5-megapixel camera.

Tumor Chunk Culture

Mammary tumors from female MMTV-*neu* transgenic mice were resected and transferred into a petri dish, rinsed in ice-cold PBS, and minced into tumor chunks (~3 mm^3) using a sterile scalpel. Tumor chunks were then washed three times in PBS prior to being seeded into 12-well tissue culture plates and cultured in 1.5 ml of serum-free Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA) for 24 hrs at 37°C in a 5% CO_2 humidified atmosphere. The tumor chunks were treated with PRL or G129R for 24 hrs. Experiments were terminated by washing the tumor chunks with ice-cold PBS supplemented with 0.4 mM sodium orthovanadate. The tumor chunks were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% (v/v) NP-40) supplemented with protease inhibitors (100 mM sodium fluoride; 2 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 1 mM sodium orthovanadate; 5 µg/ml aprotinin; 5 µg/ml leupeptin) at a concentration of 250 mg/mL and homogenized using an electric homogenizer. Lysates were harvested into microcentrifuge tubes, placed on ice for 15 min and centrifuged at 15,000 ·g for 15 min at 4ºC. Supernatants were collected and the protein content was determined using Coomassie Plus Protein Assay reagent.

Primary Tumor Cell Culture

Mammary tumors from female MMTV-*neu* transgenic mice were resected and transferred into a petri dish before they were rinsed in ice-cold phosphate buffered saline (PBS). One portion of the tumor was made into tumor chunks as described above. The second portion of tumor was minced into paste with a sterile scalpel in a petri-dish, treated with 0.25% trypsin-EDTA (Invitrogen) at 37°C for 30 min with constant shaking, and filtered through a 100-µm cell strainer. The single cell suspension was centrifuged at 800g for 10 min and resuspended and incubated in ACK Lysing Buffer (Lonza, Walkersville, MD) for 10 min. The cell suspension was filtered through a 40-µm strainer and centrifuged at 300g for 10 min. The cell pellet was washed with PBS and resuspended in DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen) and seeded (1 x 10^6 cells/well) into 6-well tissue culture plates. The cells were monolayer cultured for 24 hrs at 37°C in a 5% CO_2 humidified atmosphere. The cells were treated with PRL or G129R for 24 hrs after the depletion in DMEM supplemented with 0.5% CSS for 1 hr at 37°C. Experiments were terminated by washing the cells with PBS supplemented with 0.4 mM sodium orthovanadate and resuspending them in lysis buffer supplemented with protease inhibitors. Cell lysates were harvested into microcentrifuge tubes, placed on ice for 15 min and centrifuged at 15,000g for 15 min at 4ºC. Supernatants were collected and the protein content was determined using Coomassie Plus Protein Assay reagent (Thermo Scientific, Rockford, IL).

Co-culture

For direct co-culture, MCNeuA cells were mixed with various ratios of N202Fb3 CAFs or with a 4:1 ratio of NTG-MEFs or NEU-MEFs; SK-BR-3 cells were mixed at a 4:1 ratio with N202Fb3, NTG-MEFs, NEU-MEFs, or human CAFs. The cells were seeded (1×10^6 cells/well) in 6-well tissue culture plates and incubated for 24 hrs. The cells were serum-starved in DMEM supplemented with 0.5% charcoal stripped serum (CSS) for 1 hr prior to treatment with G129R or PRL for 1, 6 or 24 hrs. Experiments were terminated by washing the cells with PBS supplemented with 0.4 mM sodium orthovanadate and resuspending them in lysis buffer supplemented with protease inhibitors. Cell lysates were scraped off with a cell lifter, harvested into microcentrifuge tubes, placed on ice for 15 min and centrifuged at 15,000g for 15 min at 4°C. Supernatants were collected and the protein content was determined using Coomassie Plus Protein Assay reagent.

Transwell Co-culture

The experiment was carried out in 6-well cell culture plates using transwell inserts with 0.4 µm polycarbonate membranes (Corning, Corning, NY) (see fig.8). 8×10^5 MCNeuA epithelial cells were seeded in the bottom chamber and 2×10^5 N202Fb3 CAFs were seeded on the insert. The cells were incubated in 2.5 ml (per well) of DMEM supplemented with 10% FBS at 37°C in a 5% CO_2 humidified atmosphere. After serum starved in 2 ml of DMEM supplemented with 0.5% CSS, cells were treated with G129R or PRL for 24 hrs. Experiments were terminated by removing the inserts and washing the

cells in the bottom chamber with PBS supplemented with 0.4 mM sodium orthovanadate and resuspending them in lysis buffer supplemented with protease inhibitors. Only the epithelial cells in the bottom chamber were scraped off with a cell lifter, harvested into microcentrifuge tubes, placed on ice for 15 min and centrifuged at 15,000g for 15 min at 4ºC. Supernatants were collected and the protein content was determined using Coomassie Plus Protein Assay reagent.
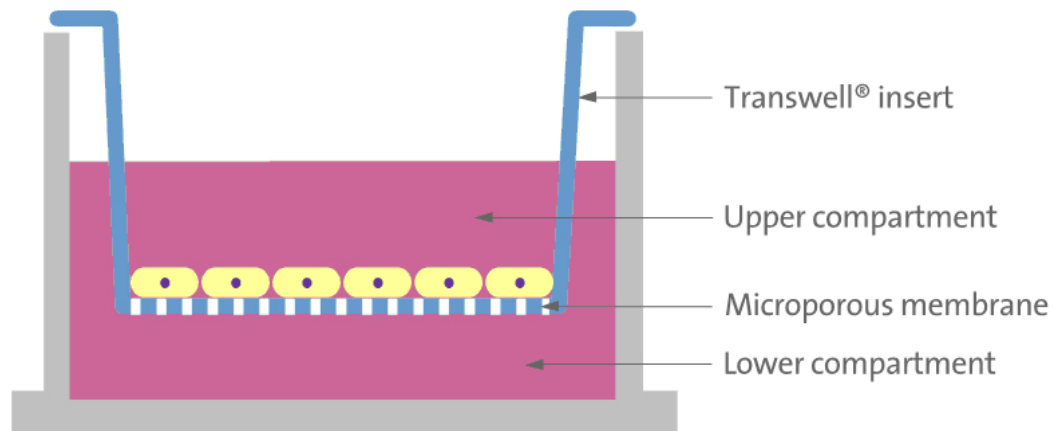
Fig. 3.3   Illustration of transwell culture apparatus

The bottom of the transwell insert is attached with a piece of 10 µm thick translucent polycarbonate membrane. The membrane is featured with micropores with a density of $1x10^5$ per cm^2 and a size of 8 µm. The hanging design keeps the Transwell$^®$ membrane about a millimeter off the bottom of the well. This prevents co-cultured cell monolayers in the bottom of the well from being scratched or disturbed when the insert is moved. Windows or openings in the sides of the Transwell insert allow access to the lower compartment (see Transwell® manual).

Fibroblast Debris Co-culture

2 x 10^5/well N202Fb3 CAFs were seeded in 6-well culture plates and incubated in DMEM supplemented with 10% FBS at 37°C in a 5% CO_2 humidified atmosphere before being harvested by scraping cells in PBS. The cells were homogenized with a Potter-Elvehjem homogenizer, and centrifuged at 500g for 5 min at 4°C. Insoluble components containing cell membrane debris were collected and mixed with MCNeuA cells at various ratios and seeded (8 x 10^5 MCNeuA cells/well) into 6-well cell culture plates. The cells were incubated in 2 ml (per well) of DMEM supplemented with 10% FBS at 37°C in a 5% CO_2 humidified atmosphere. Experiments were terminated by washing the cells with PBS three times to make sure all the cell debris was removed. Cells were then suspended in lysis buffer supplemented with protease inhibitors. Cell lysates were scraped off with a cell lifter, harvested into microcentrifuge tubes, placed on ice for 15 min and centrifuged at 15,000g for 15 min at 4°C. Supernatants were collected and the protein content was determined using Coomassie Plus Protein Assay reagent.

Preparation of Tissue and Cell Lysate

All lysate was prepared in RIPA buffer (50 mM Tris·HCl, pH 7.4; 150 mM NaCl; 1% NP40; 0.25% Na-deoxycholate; 1 mM EDTA, pH 8.0) supplemented with 1x protease and 1x phosphatase inhibitors (78430 and 78428; Thermo Scientific). Tumor chunks were transferred to tubes, weighed, and suspended in lysis buffer at a concentration of 250 mg/mL and homogenized with a Polytron homogenizer. Cells

cultured as monolayers in 6-well plates were lysed in 0.2 ml of lysis buffer. Clarified lysate and protein content were prepared as described previously [250].

Immunoprecipitation of Prolactin Receptors

Cells were lysed in 1mL of modified RIPA Buffer to which protease inhibitors were added. Cells lysate was collected into microcentrifuge tubes, centrifuged at 18,000g for 30 min and the supernatant was subject to immunoprecipitation. The concentrations of the clarified cell lysates were determined using the Coomassie Plus Protein Assay reagent with BSA standards (Thermo Scientific). Approximately 1 mg of cell lysate was incubated with 5 µg of rabbit anti-PRLR (M170) (sc-30225, Santa Cruz Biotechnology) and 25 µl of protein G-sepharose (GE Healthcare Sciences) overnight at 4ºC on a slowly rotating wheel. Immunoprecipitates were washed once in 1 ml of washing buffer (50mM HEPES, pH7.5; 150mM NaCl; 5mM EDTA; 50mM Tris-HCl, pH7.5; 0.05% Triton X-100). The immunoprecipitate was collected by centrifugation, washed, and resuspended in sample buffer (1%SDS; 100mM DTT; 50Mm Tris, pH7.5; 0.05% bromophenol blue). Whole cell lysate and immunoprecipitate were resolved by SDS-PAGE and western blotted for PRLR as described below.

SDS-PAGE and Western Blot Analysis

Tissue or cell lysates (30-60 µg/well) were mixed with Laemmli buffer (4.4 ml 0.5 M Tris, pH 6.8; 4.4 ml Glycerol; 2.2 ml 20% SDS; 0.5 ml 1% Bromophenol Blue; 0.5 ml Beta-mercaptoethanol) and heated to 80-100ºC for 5 min prior to being loaded on 4-

15% gradient polyacrylamide gels (Bio-Rad, Hercules, CA). The gels were run at 100 V for 2 hrs (or until bromophenol blue dye front reaches the bottom of the gel) in the Bio-Rad Mini-Gel Box system with running buffer (30.3 g (0.25 M) Tris Base, 144 g (1.92 M) Glycine, 10 g (1%) SDS, 1000 ml ddH2O Dilute 1:10 with ddH2O. pH will be 8.3). The gels then were placed in a transfer sandwich (black side, fiber pad, filter paper, gel, nitrocellulose membrane, filter paper, fiber pad, and red side). Any bubbles were rolled out after each layer. The transfer sandwich was run in the Bio-Rad transblot system at 4°C using an ice pack with transfer buffer (28.8g glycine, 6.04g Tris base, 100ml methanol, 1.6L double distilled water). Running time was 12 W for 2 hr.

After the transfer, the membranes (facing up) were blocked with 5% nonfat dry milk in TBS-T (960 mL distilled water, 30 mL 5 M NaCl, 10 mL 1 M Tris-HCl, 0.5 mL 100% Tween 20) pH 7.4 (1g/20 mL) for 1 hr., on the shaker, at room temperature. The blots were then incubated overnight with a 1:1000 dilution of rabbit anti-phospho-Neu (sc-12352; Santa Cruz Biotechnology, Santa Cruz, CA), a 1:1000 dilution of rabbit anti-Neu (sc-284, Santa Cruz Biotechnology), a 1:1000 dilution of rabbit anti-PRLR (sc-20992, Santa Cruz Biotechnology), a 1:10,000 dilution of mouse anti-β-actin (A1978; Sigma-Aldrich), or a 1:1000 dilution of mouse anti-β-tubulin (sc-55529; Santa Cruz Biotechnology). Subsequently, membranes were washed with deionized water and TBS-T respectively before the incubation with secondary incubation using either a 1:2,000 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Bio-Rad). Western blot detection was carried out using ECL detection reagent (GE Healthcare Sciences, Piscataway, NJ) with blots being incubated in ECL solution for 1

min. Blots were imaged for 10 min and analyzed with the FluorChem Q Imaging System and AlphaView Q software (Cell Biosciences, Santa Clara, CA).

Biopsy and Treatment of Spontaneous Mammary Tumors in Female MMTV-*neu* mice

Tumor-bearing MMTV-*neu* female mice were anesthetized. Following anesthetization, a small incision was made next to the tumor and a piece (~0.05-0.2 g) of tumor was excised and frozen on dry ice. The incision was closed with 9 mm autoclips (Clay Adams-Becton Dickinson and Company, Sparks, MD) and the mice were allowed to recover from surgery for 48 hrs. The mice were treated with Vehicle or G129R for various intervals of time. Approximately 24 hrs after the final injection, mice were euthanized and tumors were resected and frozen on dry ice. Pre- and post-treatment biopsies were lysed and blotted as described above.

3D Culture

Matrigel was thawed out at 4°C overnight. 20 ml matrigel was used to coat the prechilled 12-well plates to form a thin layer at the bottom. Plates were incubated for 15–30 min at 37°C to allow the gel to solidify (but do not let it overdry). Cells (1 x 10^5 cells/well) were trypsinized from a monolayer to a single-cell suspension and pelleted by centrifugation at 115g before resuspended in the mixture of DMEM (500ul/well) and matrigel (120 µl/well). The mixture of cells was placed onto the coated surface and was allowed to settle and attach to the matrigel at the bottom for 30 min at 37º C. Another 500

µl DMEM and 120 µl EHS were added on top of the gel and the culture was maintained

for 4 days before treatments were carried out (see Fig. 3.4).
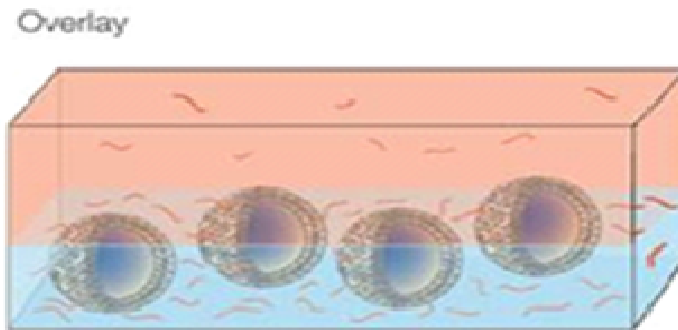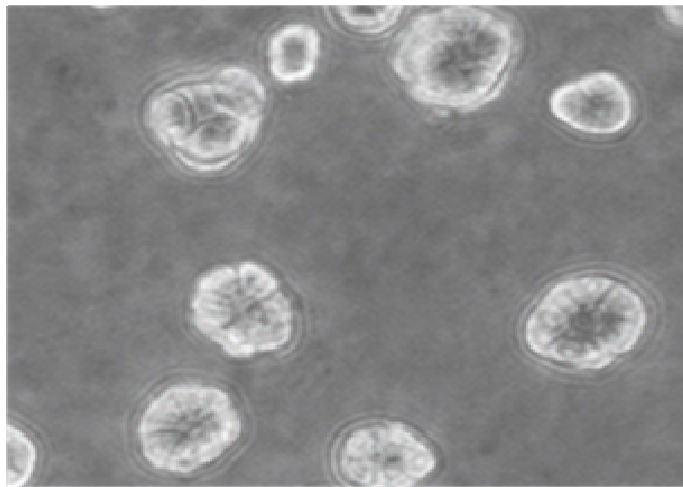
Fig. 3.4   Illustration of three-dimensional cell culture in matrigel

(A) In the overlay method, the ECM is first cast to form a gelled bed measuring 1 mm in thickness. Epithelial cells are seeded onto this bed as a single-cell suspension in culture media. (B) Microscopic image of tumor cells forming spheres in matrigel [72].

Statistical Data Analyses

For immunoblots, the Blots were imaged and analyzed with the FluorChem Q Imaging System and AlphaView Q software (Cell Biosciences, Santa Clara, CA). Densitometric values of the net intensity were based on replicates of quantified protein bands normalized to the β-actin or β-tubulin levels and the results were expressed as the percentage change of the mean $\pm$ SD. Statistical differences between the groups were determined using Student's t-test and a two-tailed distribution with unequal variances. A value of $P < 0.05$ and $P < 0.001$ was considered significant and very significant, respectively.

CHAPTER FOUR

RESULTS

Neu Activation Level is Not Related to Tumor Size or Tumor Onset in Female MMTV-*neu* Transgenic Mice

As tumor size and tumor onset differ among individual mice, the p-Neu status was investigated in mammary tumors, normal mammary glands, metastatic lungs and normal lung tissues in MMTV-*neu* transgenic mice. In the first group, 10 mice of the same age with mammary tumors of different sizes were selected. Tumors were resected and weighed. Healthy and tumor harboring lung tissue were also removed and made into lysates. No correlation was detected between tumor size and p-Neu level (only representative data are shown). Also, p-Neu levels in metastatic lung tumors did not appear to be consistent with that in original mammary tumors. Neu was minimally expressed and no activation was observed in healthy lung tissues (Fig. 4.1). In the second group, 10 mice with the same tumor onset were selected. Normal mammary glands along with mammary tumors in various sizes were resected from female MMTV-*neu* mice. No correlation was observed between latency and p-Neu level (Only representative data are shown). No Neu activation was detected in mammary glands. The third group was comprised of eight female MMTV-*neu* mice with tumors in about the same size. As expected, the Neu activation levels fluctuated despite the uniform size of tumors. These results suggested that the breast tumor is comprised of such a heterogeneous population that HER2/Neu may be not the only driving force in tumor growth. And this indicated the

possible crosstalk between HER2/Neu and other growth factor receptors such as PRLR,

ER or PR.
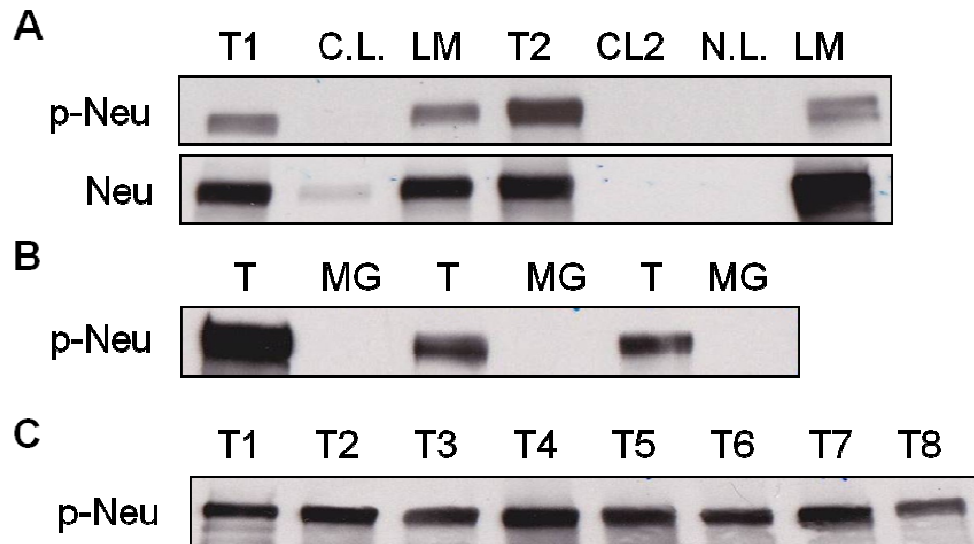
Fig. 4.1   Tyrosine phosphorylation of Neu (p-Neu) does not correlate with tumor size or tumor onset in female MMTV-*neu* transgenic mice

Mammary tumors of varying size and metastatic lung tumors along with normal surrounding tissue were resected from ten mice of the same age and made into lysates. (A) Western blot analysis shows no correlation between tumor size and p-Neu level (only representative data are shown). p-Neu levels in metastatic lung tumors (LM) did not correlate with original mammary tumors (T). Normal lung tissue (NL) expresses minimal Neu, if any. (B) Ten mice with the same tumor latency were selected. Normal mammary glands (MG) along with mammary tumors (T) in various sizes were resected from female MMTV-*neu* mice. Western blot analysis suggests that no correlation between latency and p-Neu level (only representative data are shown). No p-Neu was detected in normal mammary glands. (C) Eight female MMTV-*neu* mice with tumors of about the same size were resected and lysed. Western blot shows the p-Neu levels fluctuate despite the uniform size of tumors.

Primary Tumor Cells Isolated from Mammary Tumors in Female MMTV-*neu* Transgenic Mice are Mostly Epithelial-Origin and Express Prolactin Receptors

To further investigate mammary tumors from female MMTV-*neu* transgenic mice, tumors were allowed to grow to a certain size (1.5 cm in diameter) and were resected with sterile scalpels. Samples were minced and processed into cell suspensions. A portion of primary cell suspensions were incubated with anti-EpCAM primary antibody conjugated with PE fluorochrome. As an epithelial cell marker, EpCAM was found to be expressed in more than 99% primary tumor cells using FACS analysis (Fig. 4.2). Therefore, we concluded that the primary tumor cells were mostly epithelial-origin. Another finding was that near 94% primary tumor cells expressed PRLR which laid the groundwork for PRL signaling study in this project.
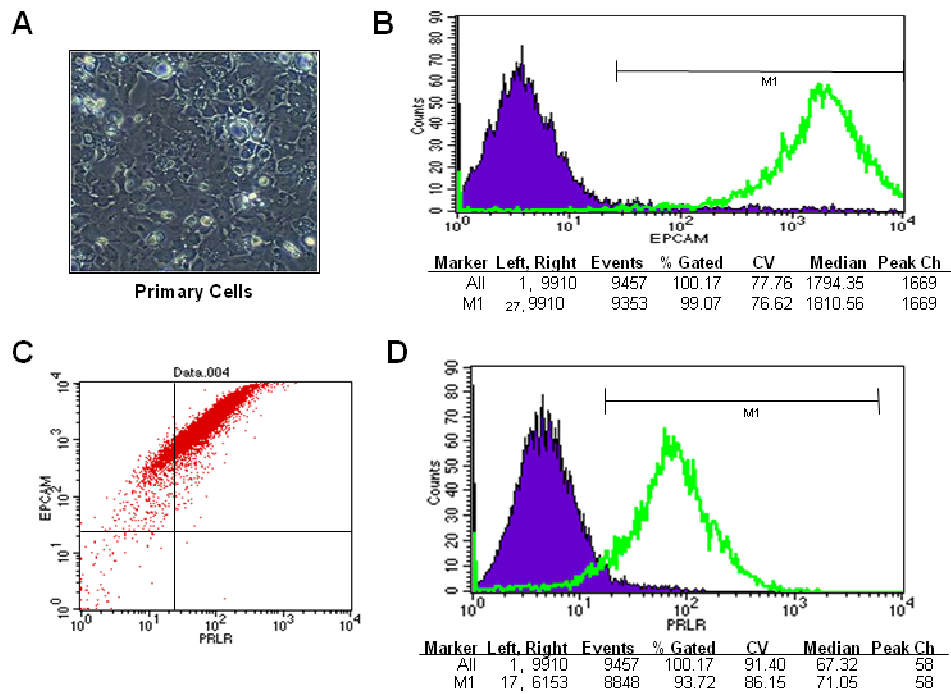
Fig. 4.2 Primary tumor cells isolated from mammary tumors in female MMTV-*neu* transgenic mice are mostly of epithelial-origin and express prolactin receptors

Tumors were allowed to grow to a proper size (1.5 cm in diameter) and were resected with sterile scalpels. (A) Samples were minced and processed into cell suspensions. (B) A portion of primary cell suspensions were incubated with anti-EpCAM (an epithelial cell component) primary antibody conjugated with PE fluorochrome. FACS analysis indicates EpCAM is expressed in more than 99% primary tumor cells. (C and D). Another portion of primary cell suspensions were incubated with anti-PRLR primary antibody conjugated with FITC fluorochrome. FACS analysis shows up to 94% primary tumor cells express PRLR.

Inhibition of p-Neu by G129R is Dependent Upon Tumor Microenvironment in MMTV-*neu* Tumors

Previous studies suggested that p-HER2 phosphorylation was enhanced by PRL [306] and inhibited by G129R [250] in T-47D and BT-474 human breast cancer epithelial cells. In this project, the effects of PRL and G129R upon p-Neu were examined in a mouse model of HER2-positive breast cancer. Tumors were resected from female MMTV-*neu* mice and processed into cell suspensions and tumor chunks. The primary cell suspensions prepared from the tumor chunks were found to be almost entirely epithelial by flow cytometry (>99% EpCAM positive cells; mean fluorescence intensity >1700), which was visually confirmed upon examination of the cells 24 hrs after seeding on tissue culture plates (Fig. 4.2A). Both primary cells and tumor chunks were depleted in DMEM supplemented with 0.5% charcoal stripped serum (CSS) and treated with G129R (10 and 50 µg/ml) or PRL (0.5 and 1 µg/ml) for 24 hrs before harvested. In contrast to the previous experiments conducted using T-47D and BT-474 cells, G129R had little effect upon p-Neu level in the primary epithelial cells isolated from MMTV-*neu* tumors when cultured in monolayer (Fig. 4.3B); however, G129R inhibited p-Neu (Fig. 4.3C) and PRL induced p-Neu (Fig. 4.3D) in a dose-dependent manner when the primary epithelial cells from the same tumors were maintained as tumor chunks. This obvious discrepancy between primary cell culture and tumor chunks suggested the inhibition of p-Neu by G129R is dependent upon tumor microenvironment.

A

Tumor Chunks

B
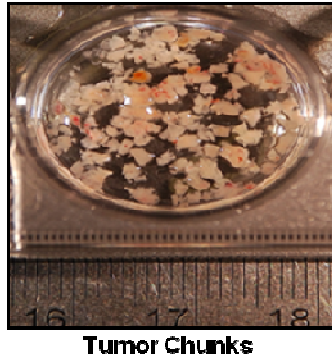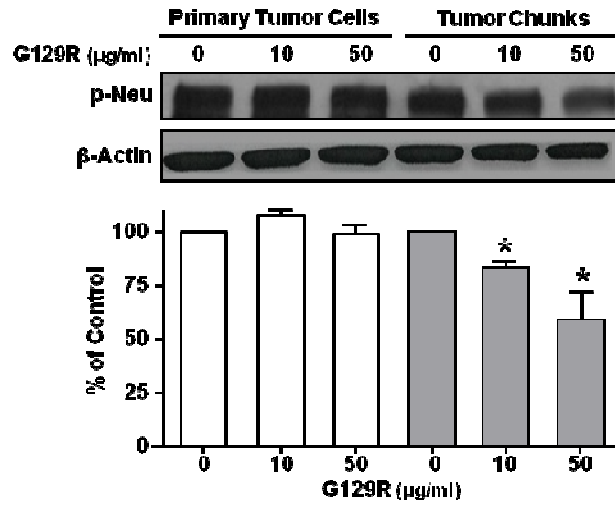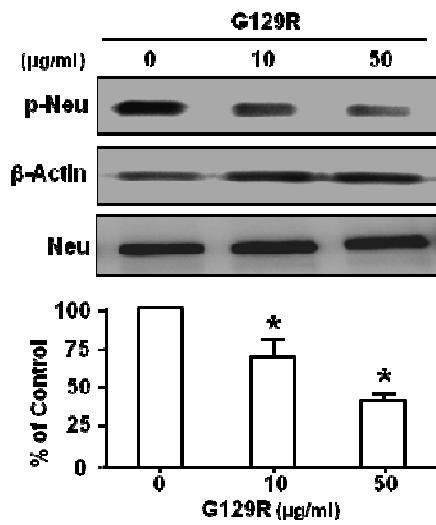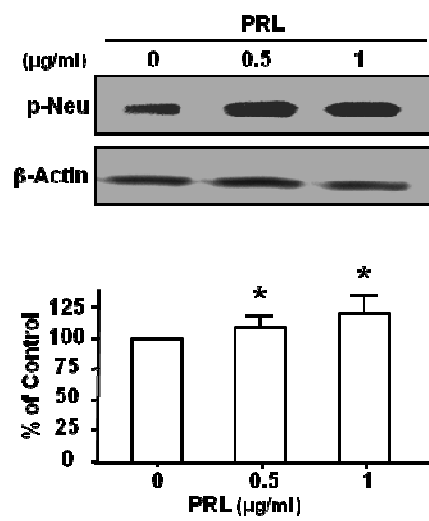
Fig. 4.3   Inhibitory effect of G129R upon p-Neu is dependent upon tumor microenvironment

Mammary tumors from MMTV-*neu* transgenic mice were split in two portions. One portion was digested with trypsin for monolayer culture in DMEM + 10% FBS. (A) The other portion of the tumor was dissected into 3 mm^3 chunks and cultured in serum-free DMEM. (B) Western blot analysis shows that G129R treatment (10 μg/ml, 50 μg/ml, 24 hrs) has little effect on primary cells in monoculture. In contrast, p-Neu is abrogated in tumor chunks in a dose-dependent manner. (C) G129R and (D) PRL modulate the phosphorylation of Neu in tumor chunks. All the experiments were repeated three times. Paired t test was the method used for statistical analysis. "*" means p value is less than 0.05, "**" means p value is less than 0.01.

In the Context with Tumor Microenvironment, G129R Exerts Additive Effect When Combined with Lapatinib

Previously, we reported that G129R may exert an additive effect on the inhibition of HER2 over-expressing breast cancer cell lines when combined with trastuzumab. G129R was once again combined with an anti-HER2/Neu reagent, lapatinib. Lapatinib is a small molecule tyrosine kinase inhibitor that blocks the kinase activity of HER1 and HER2 [204]. Data shows that lapatinib drastically reduces HER2 phosphorylation in SK-BR-3 (Fig. 4.4A) and BT-474 (Fig. 4.4B) human breast cancer cell lines and Neu phosphorylation in mouse mammary tumor chunks in a dose-dependent manner with maximal suppression being observed at 10 µM (Fig. 4.4 and 4.5). Lapatinib was clearly more potent than G129R in the inhibition of p-HER2/Neu. Tumor chunks treated with lapatinib and G129R significantly reduced Neu activation, with the higher dose (10 µM lapatinib + 50µg/ml) almost completely nullifying p-Neu (Fig. 4.5). The combination of the two drugs down-regulated Neu phosphorylation on a greater degree than either single drug treatment.
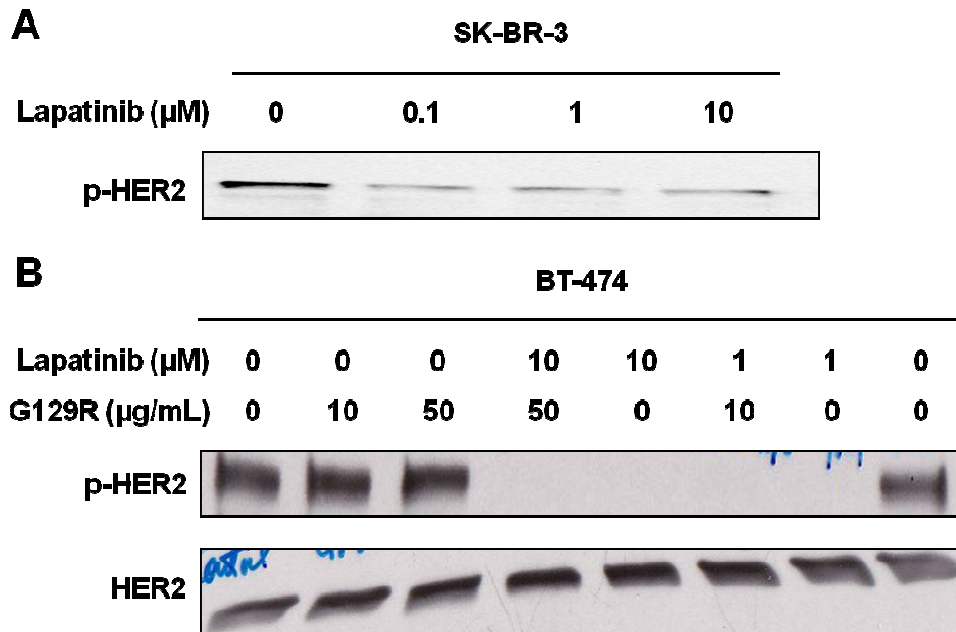
Fig. 4.4   Lapatinib inhibits HER2 phosphorylation in SK-BR-3 and BT-474 cells

SK-BR-3 and BT-474 breast cancer cell lines were monolayer cultured in RPMI 1640 medium supplemented with 0.5% CSS before they were treated for 24 hrs. (A) Western blot analysis indicates that lapatinib (0.1, 1, 10 µM) drastically reduces HER2 activation in SK-BR-3 cells. (B) Western blot analysis indicates that lapatinib (10 µM) has an inhibitory effect on HER2 activation in BT-474 cells in the absence or presence of G129R; whereas, G129R (10, 50 µg/ml) alone fails to reduce p-HER2 levels.

A

Tumor chunk				
Lapatinib (µM)	0	1	10	0	0
G129R (µg/mL)	0	0	0	10	50
p-NEU					
NEU					

B

Tumor chunk					
Lapatinib (µM)	0	0.1	1	1	10	10
G129R (µg/mL)	0	0	0	10	0	50
p-NEU						
NEU						
β-actin						
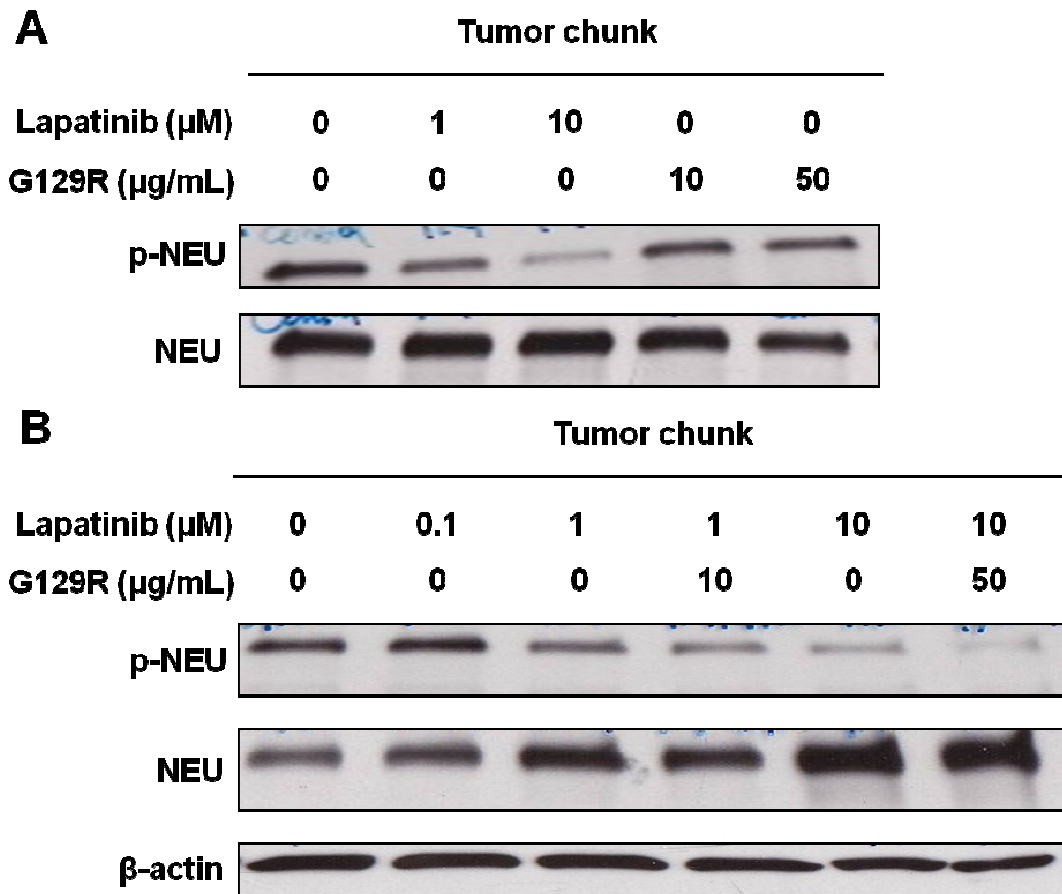
Fig. 4.5   In the context with tumor microenvironment, G129R exerts a additive effect when combined with lapatinib

G129R and lapatinib are used in tumor chunk model. (A) Western blots show lapatinib drastically reduces Neu phosphorylation in tumor chunks in a dose-dependent manner with a maximal suppression dose at 10 µM. Lapatinib was clearly more potent than G129R in the inhibition of p-Neu. (B) Tumor chunks treated with lapatinib combined with G129R (1 µM lapatinib + 10 µg/ml G129R and 10 µM lapatinib + 50 µg/ml G129R) significantly reduced Neu activation, with the latter almost completely eliminatingp-Neu.

Mouse and Human Breast Cancer Cell Lines Express Prolactin Receptors

To make sure that the cell lines used in this study expressed PRLR, PRLR was examined in multiple breast cancer cell lines including T-47D, BT-474, SK-BR-3, and BT-483 human cell lines (Fig.4.6) and MCNeuA and N202Fb3 mouse cell lines (Fig. 4.7A). MCNeuA and N202Fb3 cells are epithelial and fibroblast cell lines, respectively, derived from the same mammary tumor of a female MMTV-*neu* transgenic mouse. The cancer cell lines were seeded on glass slides and fixed with 10% neutral buffered formalin before being permeabilized in 10% Triton X-100. Cells were treated with anti-PRLR (H300) antibody and the UltraVision ONE Detection System HRP Polymer and DAB Plus Chromogen kit was used for immunohistochemistry. HeLa cells were used as a negative control. As expected, PRLRs were expressed in all of the human breast cancer cell lines. Detection of PRLR in MCNeuA and N202Fb3 cells was examined by both immunohistochemistry and immunoprecipitation followed by western blotting. Immunohistochemically, MCNeuA exhibited higher expression of PRLR compared with N202Fb3 cells (Fig. 4.7A). For immunoprecipitation, approximately 1 mg of cell lysate was incubated with 5 µg of rabbit anti-PRLR (M170) (This antibody was raised against mouse PRLR) and 25 µl of protein G-sepharose. Both cell lines expressed low level of PRLR compared with the expression level in T-47D cells (Fig. 4.7B). T47D, BT474, SK-BR-3, BT483 and MCNeuA are breast cancer epithelial cell lines known for the overexpression of HER2/Neu. As expected, PRLR were co-expressed with HER2/Neu in all of these epithelial cells.
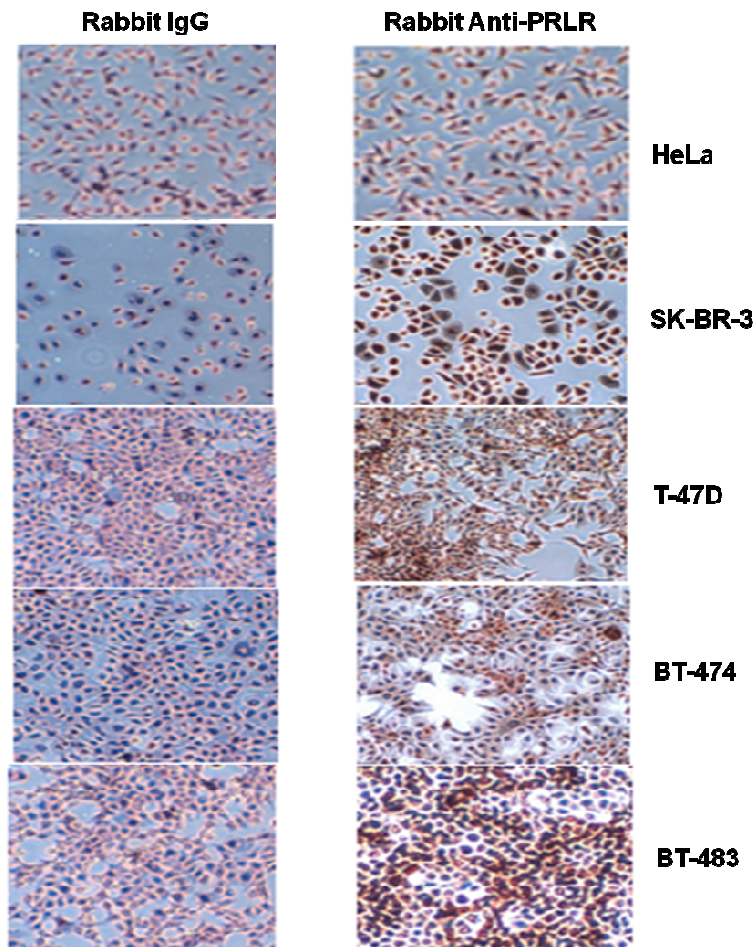
Fig. 4.6   Mouse and human breast cancer epithelial cell lines express prolactin receptors at different levels

SK-BR-3, T-47D, BT-474, BT-483 breast cancer cells were seeded on glass slides and fixed with 10% neutral buffered formalin and permeabilized in 10% Triton X-100. Cells were treated with anti-PRLR (H300) antibody and the UltraVision ONE Detection System HRP Polymer and DAB Plus Chromogen kit was to perform immunohistochemistry.   HeLa cells were used as a negative control. Immunohistochemistry shows that all the human cell lines tested express PRLR at different levels.

A

IHC: PRLR

MCNeuA & N202Fb3

B

WB: PRLR   IP: PRLR + WB: PRLR

| T-47D | N202Fb3 | MCNeuA | T-47D | N202Fb3 | MCNeuA |

75 kDa —
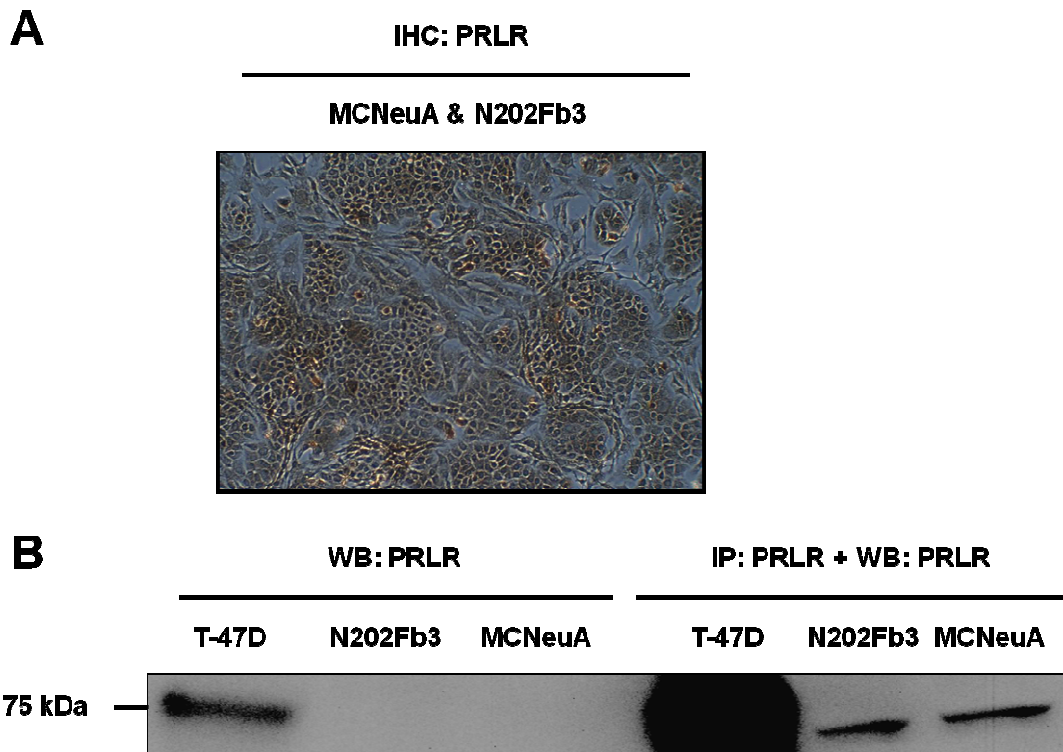
Fig. 4.7   MCNeuA and N202Fb3 cells express prolactin receptors at different levels

(A) MCNeuA and N202Fb3 cells were seeded together on glass slides and fixed with 10% neutral buffered formalin before being permeabilized with10% Triton X-100. Cells were treated with anti-PRLR antibody raised against mouse PRLR and the UltraVision ONE Detection System HRP Polymer and DAB Plus Chromogen kit was used for immunohistochemistry (IHC). IHC shows that both cell lines are able to express PRLR. MCNeuA cells express higher levels of PRLR than N202Fb3 cells. (B) Western blot analysis of PRLR in T-47D (positive control), MCNeuA, and N202Fb3 cells using 40 µg of cell lysate and of immunoprecipitated PRLR starting with 1 mg of cell lysate. Data suggest that PRLR level in mouse cell lines is much lower than that in the human cell line, T-47D.

Co-cultured Breast Cancer Epithelial Cells and CAFs Exhibit Morphology Similar to Original Tumor

This co-culture experiment was conducted to test if the discrepancy observed between the response to G129R in primary tumor cells and tumor chunks is due to the absence of tumor microenvironment components. The importance of CAFs in mediating the inhibitory effect of G129R upon p-Neu was examined by directly co-culturing MCNeuA epithelial and N202Fb3 CAFs established from a spontaneous MMTV-*neu* mammary tumor. Similar to what was observed *in vivo*, microscopic images revealed that the MCNeuA epithelial cells form islets surrounded by small nest-like clusters of N202Fb3 CAFs (Fig. 4.8).

MCNeuA

Mouse IgG | Anti-E-Cadherin

N202Fb3

Mouse IgG | Anti-Vimentin

MCNeuA + N202Fb3

Mouse IgG | Anti-E-Cadherin | Anti-Vimentin
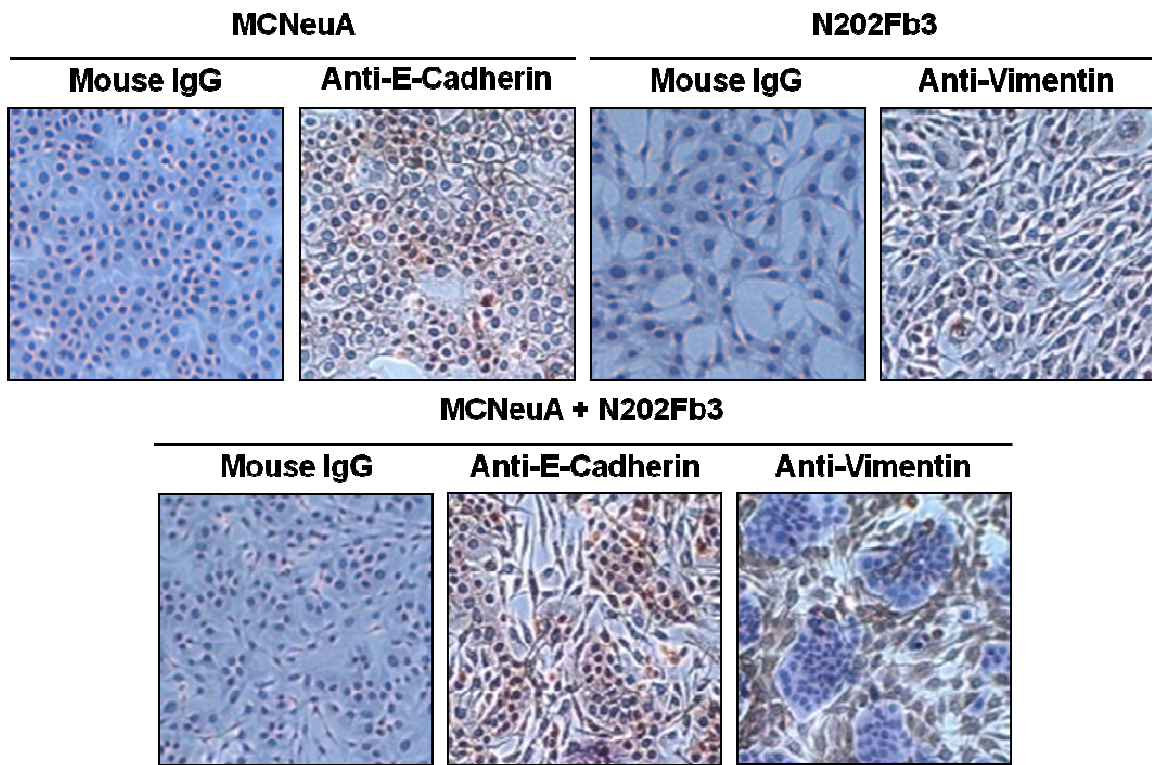
Fig. 4.8   Co-cultured breast cancer epithelial cells and CAFs exhibits morphology similar to original tumor

Microscopic images of MCNeuA (epithelial) cells and N202Fb3 (fibroblast) cells in monoculture or co-cultured at a 4:1 ratio. MCNeuA cells and N202Fb3 cells were distinguished by using immunohistochemistry for E-cadherin and vimentin, respectively.

CAFs Modulate the Effect of G129R and PRL Upon p-Neu

Treatment with G129R had a minimal effect, if any, on p-Neu when MCNeuA cells were grown alone in monolayer (Fig. 4.9); similar to what was observed with primary epithelial cells. To investigate the influence of CAFs upon p-Neu in tumor epithelial cells, direct co-culture experiments were conducted using different ratios (1:4, 1:2, 1:1, 2:1, 4:1, or 8:1) of MCNeuA cells to N202Fb3 CAFs. After 24 hrs treatment with G129R (10 µg/ml), a reduction in p-Neu was observed in cells seeded at a 2:1 and 4:1 ratio (MCNeuA:N202Fb3), with the maximum reduction in p-Neu being observed at a 4:1 ratio (Fig. 4.9). Time-course experiments revealed that the MCNeuA cells had to be co-cultured with N202Fb3 CAFs for at least 24 hrs prior to treatment in order to observe a reduction in p-Neu by G129R (10 µg/ml) (Fig. 4.10). Under similar conditions, PRL (0, 0.1, 0.2, 0.5, 1.0, or 2.0 µg/ml) increased p-Neu in MCNeuA in a dose-dependent manner (Fig. 4.10B). Fluctuations in p-Neu in response to G129R treatment were observed in different experiments. Even though the ratios and courses of incubation were identical among co-culture experiments, the nest-like structures formed between the epithelial cells and fibroblasts were often of different size and shape. Most of the variability observed in response to G129R is likely due to these obvious alterations in the interactions between the epithelial cells and fibroblasts. Different cell passage numbers and lot-to-lot variability in culture medium, serum, and G129R may have also contributed to the variability.
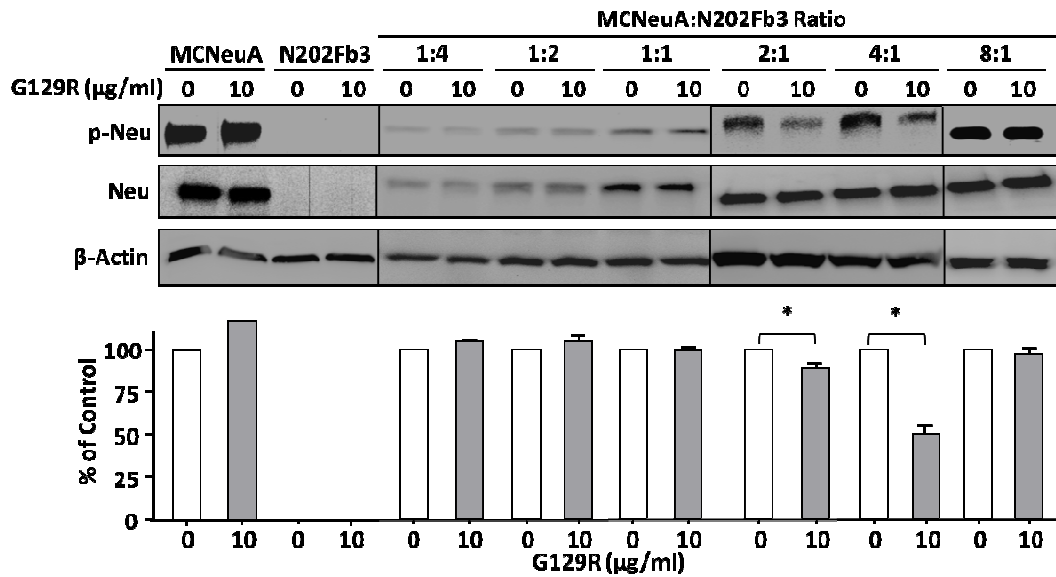
Fig. 4.9   Fibroblasts in co-culture modulate the effect of G129R and PRL upon Neu phosphorylation

Co-culture of MCNeuA and N202Fb3 cells at various ratios (1:4, 1:2, 1:1, 2:1, 4:1 or 8:1) were treated with G129R for 24 hrs. Western blot analysis shows the inhibition of p-Neu by G129R is obvious at 2:1 and 4:1 ratio. All experiments were repeated three times. Paired t test was used. "*" means p value is less than 0.05.
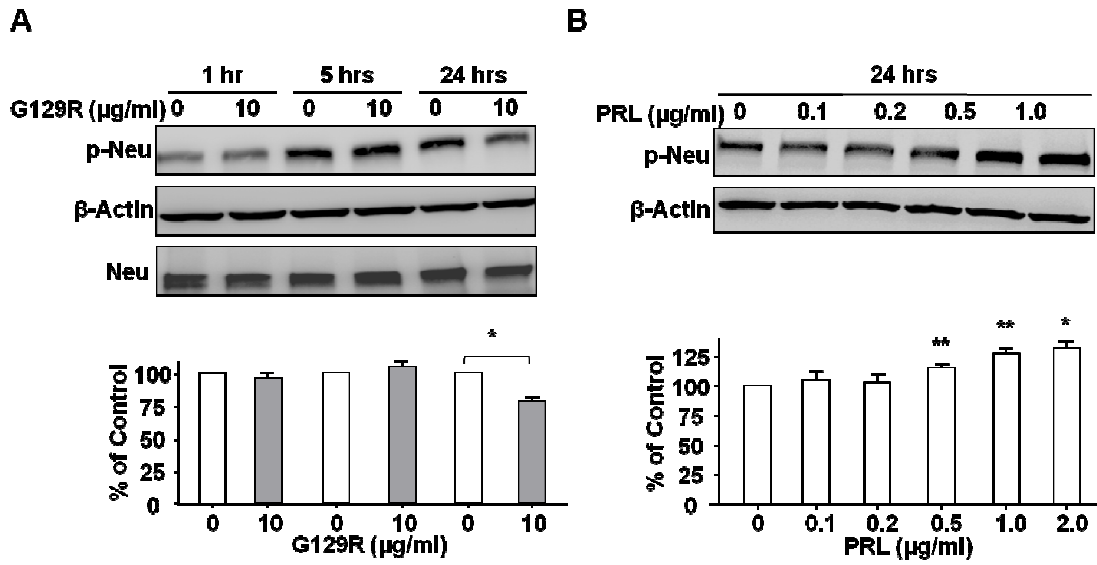
Fig. 4.10   G129R inhibits and PRL induces p-Neu in co-culture after 24 hrs in a dose-dependent manner

(A) MCNeuA and N202Fb3 cells were co-cultured at a 4:1 ratio and treated with G129R. Treatments were terminated after 1, 5, or 24 hrs. Western blot analysis shows that the maximal inhibitory effect of G129R upon p-Neu is observed at 24 hrs. (B) MCNeuA and N202Fb3 cells were co-cultured at 4:1 ratio and were treated with various concentrations of PRL for 24 hrs. Western blot results indicate that PRL increases p-Neu in a dose-dependent manner. All experiments were repeated three times. Paired t test was used. "*" means p value is less than 0.05.

In Co-culture, Normal Fibroblasts Possess Different Morphology From That of CAFs

CAFs represent a group of fibroblasts in close proximity to cancer epithelial cells in tumors. They are perpetually activated and are considered to be distinct from normal, non-activated fibroblast on many levels. To test if the observation regarding the tumor fibroblast is truly CAF specific, I introduced two types of normal fibroblasts designated as NTG-MEF and NEU-MEF, derived from embryos of non-transgenic and MMTV-*neu* transgenic mice, respectively in similar co-culture experiment setting. In contrast to CAFs co-cultured with MCNeuA cells, co-culture of NTG-MEFs and NEU-MEFs both exhibited different morphology, characterized by larger cell processes (Fig. 4.11). Of note, in the coculture of MCNeuA and NTG-MEF cells, the formation of islets of MCNeuA was much less obvious than those observed in the coculture with N202Fb3 or NEU-MEF. At this point, I am not sure if this morphological difference induced by N202Fb3 and NTG-MEF cells contributed to or determined the cross talk between the two receptors. Nonetheless, it was an interesting observation.

NTG-MEF

Anti-E-Cadherin	Anti-Vimentin	Mouse IgG

NEU-MEF

Anti-E-Cadherin	Anti-Vimentin	Mouse IgG
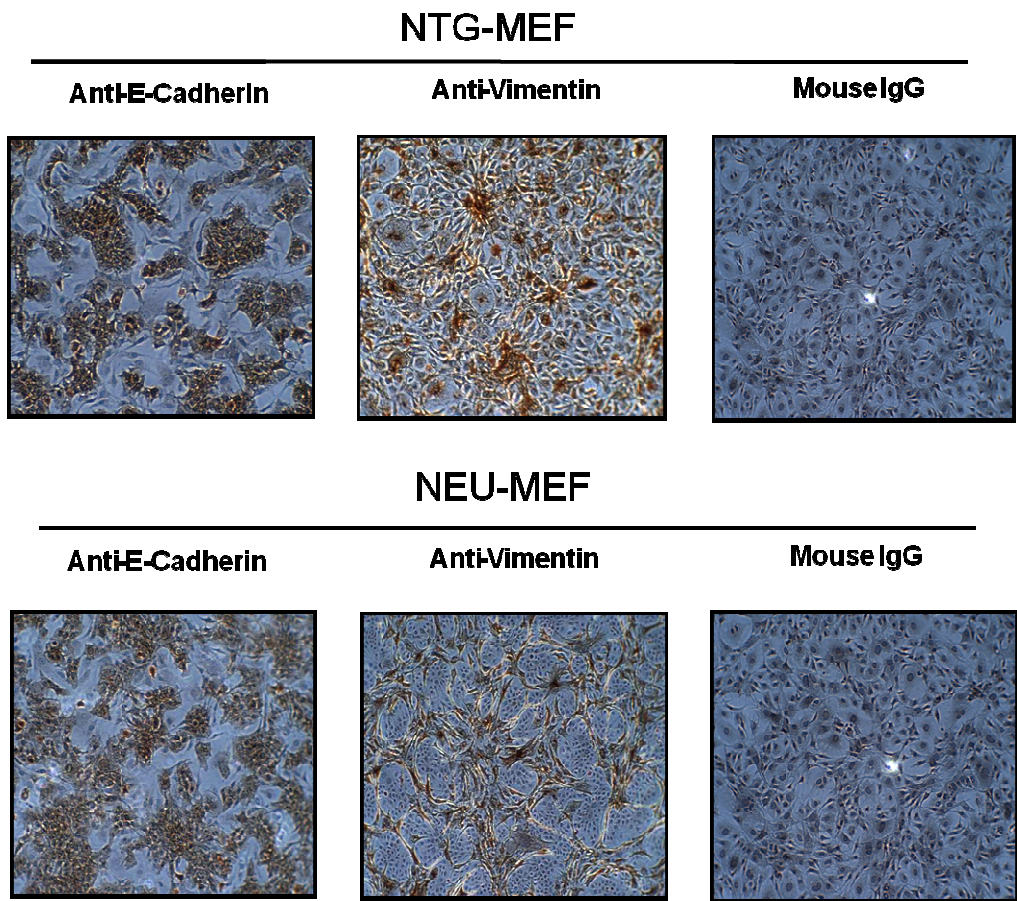
Fig. 4.11   Co-culture of breast cancer epithelial cells and non-CAFs exhibits different morphology

Microscopic images of MCNeuA (epithelial) cells co-cultured with mouse embryonic fibroblasts derived from a non-transgenic FVB mouse (NTG-MEF) or MMTV-*neu* transgenic mouse at a 4:1 ratio. MCNeuA cells and MEFs were distinguished by using immunohistochemistry for E-cadherin and vimentin, respectively.

CAFs Have Properties Not Present in Normal Fibroblasts That Modulate the Effects of G129R and PRL Upon p-Neu

Using the same direct co-culture system, it was further evaluated whether or not the inhibitory effect of G129R in MCNeuA cells which was restored by CAFs could be replaced by co-culture with normal embryonic fibroblasts i.e. CAFs were replaced with NTG-MEFs or NEU-MEFs. However, treatment with G129R (10 µg/ml) for 24 hrs reduced p-Neu in MCNeuA cells co-cultured with N202Fb3 CAFs, but not in MCNeuA cells co-cultured with either of the MEFs (Fig. 4.12). These results suggest that the modulation of G129R's inhibitory effect on pHER2 in MCNeuA cells is CAF specific.
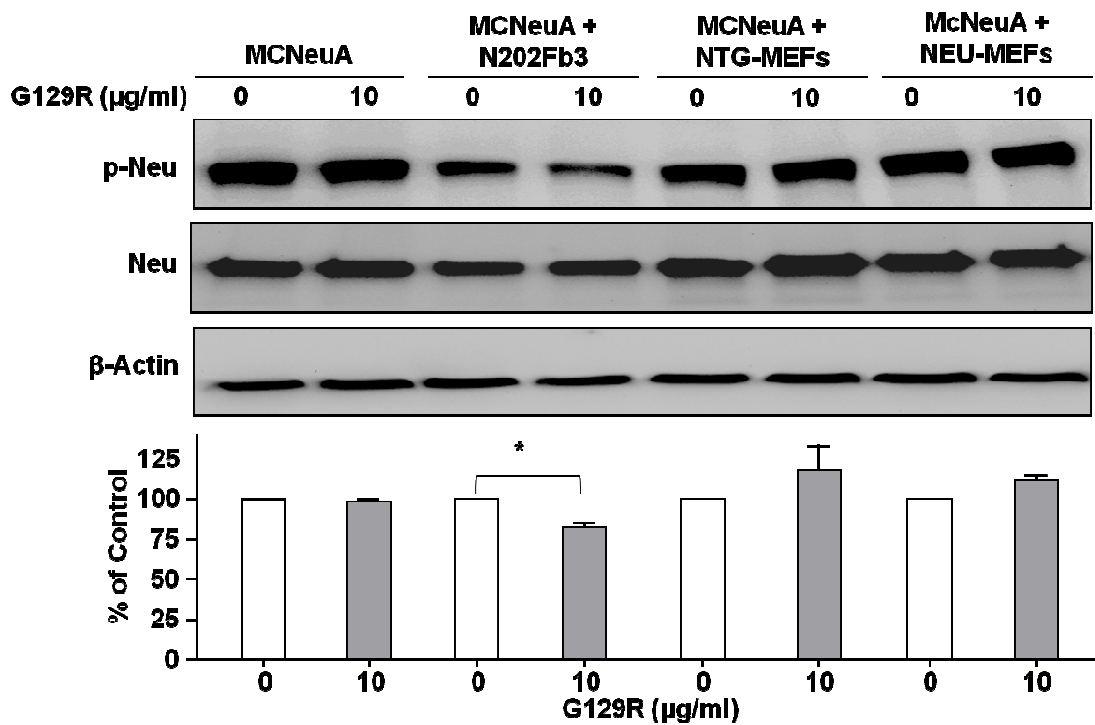
Fig. 4.12   The inhibitory effect of G129R upon epithelial cells vanishes when CAFs are replaced with normal fibroblasts in co-culture

MCNeuA cells were directly co-cultured at a 4:1 ratio with N202Fb3 CAFs or with NTG-MEFs or NEU-MEFs derived from embryos of non-transgenic or Neu transgenic FVB/N mice, respectively. The results show that G129R reduces p- Neu in MCNeuA cells only when co-cultured with N202Fb3 CAFs, but not when cultured alone or with NTG-MEFs or NEU-MEFs. All experiments were repeated three times. Paired t test was used. "*" means p value is less than 0.05.

Modulation of the Cellular Signaling by Tumor Epithelial-Stromal Interactions Require Live Stromal Cells, Not Simply Cell Membrane Components

To examine whether modulation of the cellular signaling in the epethilium requires live CAFs or mere cell membrane molecules, a predetermined amount of N202Fb3 cells were homogenized Insoluble components containing cell membrane debris were collected and incubated with MCNeuA cells at a 2:1, 4:1 and 8:1 ratio (MCNeuA:N202Fb3, before grinding). Treatment with G129R (10 µg/ml) for 24 hrs had no effect upon p- Neu (Fig. 4.13).

MCNeuA + N202Fb3 Cell Debris

	2:1		4:1		8:1	
G129R (µg/ml)	0	10	0	10	0	10
p-Neu						
β-Actin						
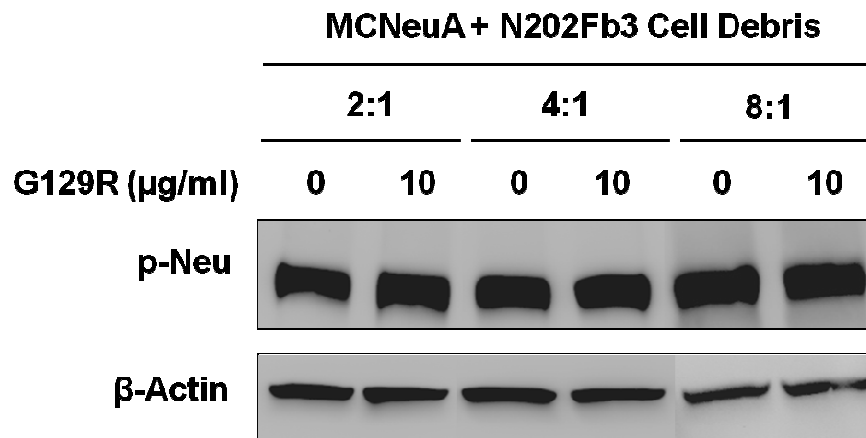
Fig. 4.13   Tumor epithelial-stromal interactions require live stromal cells, not simply cell membrane components

MCNeuA cells were cultured with insoluble components of N202Fb3 cells at a 2:1, 4:1 and 8:1 ratio (MCNeuA:N202Fb3, before homogenization) and treated with G129R for 24 hrs. Western blot results show mere cellular components of N202Fb3 cells do not sensitize MCNeuA cells to G129R.

Inhibition of p-Neu by G129R is Dependent Upon Tumor Epithelial-Stromal Interactions and Not Secreted Soluble Factors

To determine if the influence of CAFs on G129R response was mediated by direct cell-cell and/or cell-matrix contacts, or via the secretion of soluble factors, MCNeuA cells and N202Fb3 CAFs were co-cultured together or in close proximity separated by a permeable membrane. Using the transwell system, MCNeuA cells were placed in the bottom chamber and N202Fb3 CAFs were placed on the transwell insert; treatment with G129R (10 µg/ml) for 24 hrs had no effect upon p-Neu in the absence of cell-cell and/or cell-matrix contact (Fig. 4.14).
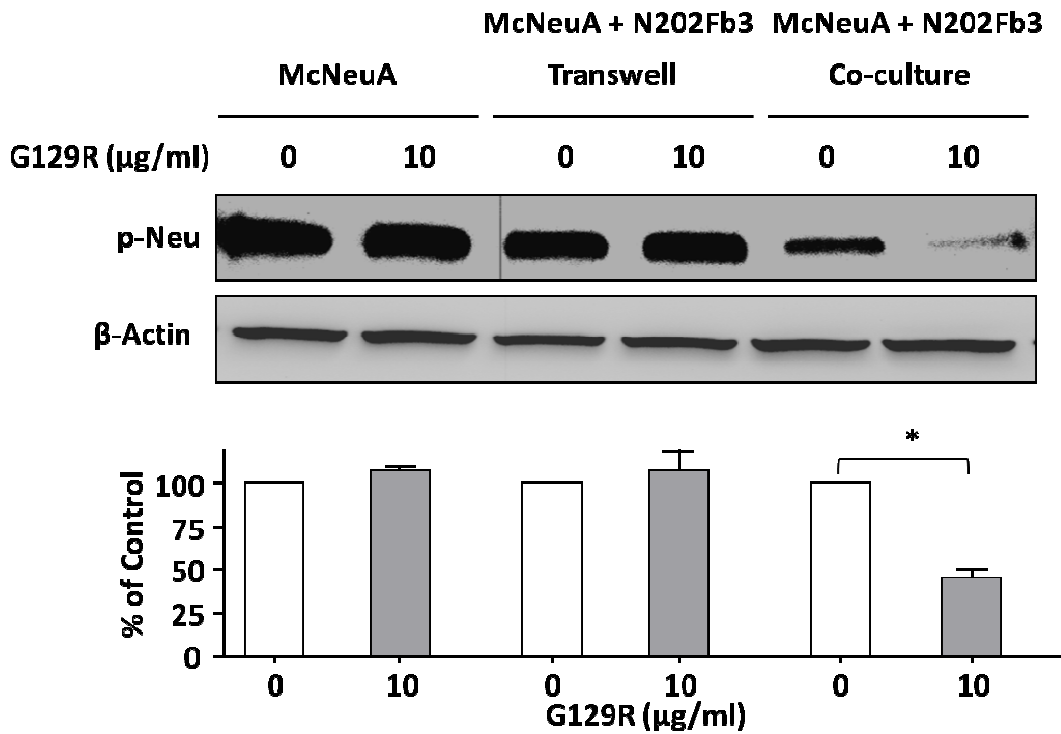
Fig. 4.14   The inhibitory effect of G129R upon epithelial cells vanishes when CAFs are separated from epithelial cells using a transwell culture system

MCNeuA cells were seeded in the bottom chamber of a 6-well transwell system and cultured with or without N202Fb3 cells on the insert at a 4:1 ratio or they were directly co-cultured with N202Fb3 cells prior to treatment with G129R for 24 hrs. Western blot results show that MCNeuA cells do not respond to G129R when cultured alone or when physically separated from N202Fb3 cells. All experiments were repeated three times. Paired t test was used. "*" means p value is less than 0.05.

G129R Reduces p-Neu Level *In Vivo*

To assess the efficacy of G129R *in vivo*, a self comparison model was developed using pre- and post-treatment tumor biopsy samples to compare the levels of p-HER2/Neu before and after treatment with G129R. Spontaneous tumors arising in female MMTV-*neu* mice were allowed to reach approximately 1 cm in diameter before tumor biopsy. Two days after biopsy, mice were treated with various doses of G129R for five or ten days. Approximately 24 hrs following the final treatment the tumors were removed. Phospho- Neu was reduced by G129R treatment in a dose-dependent manner, with a noticeable reduction seen with 5 mg/kg/day dose and maximal reduction seen with 10 mg/kg/day dose (Fig. 4.15). The inhibitory effect of G129R upon p-Neu was observed in as few as 5 days and was sustained after 10 days of treatment (Fig. 4.16).
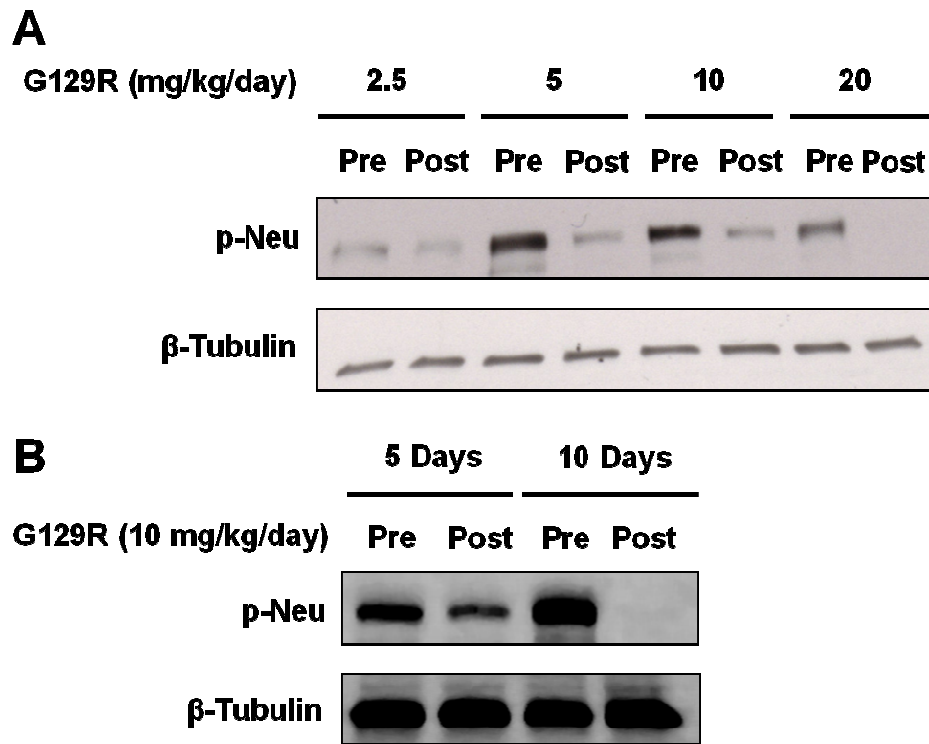
Fig. 4.15   G129R inhibits p-Neu in spontaneous MMTV-*neu* tumors *in vivo*

(A) Pre- and post-G129R treatment biopsy samples were used for dose-response analysis and p- Neu was assessed by western blot. (B) Pre- and post-G129R treatment biopsy samples were used for time-course analysis and p-Neu was assessed by western blot. All experiments were repeated three times.

Having optimized the dose of G129R (10 mg/kg/day), the response to G129R was tested on a greater number of mice. Fourteen mice with spontaneous tumors received a 5-day treatment of G129R (10 mg/kg/day, i.p.) with the goal to reduce p-Neu. Five mice were highly responsive, four were moderately responsive, and five were unresponsive, with a total response rate of 64% (9/14). Ten mice received a 10-day treatment of G129R (10 mg/kg/day, i.p.) with the goal to reduce p-Neu. Tumors from three mice were highly responsive, five were moderately responsive, and two were unresponsive, with a total response rate at 80% (8/10). A portion of the results for the 5-day and 10-day G129R treatments are shown (Fig. 4.16).
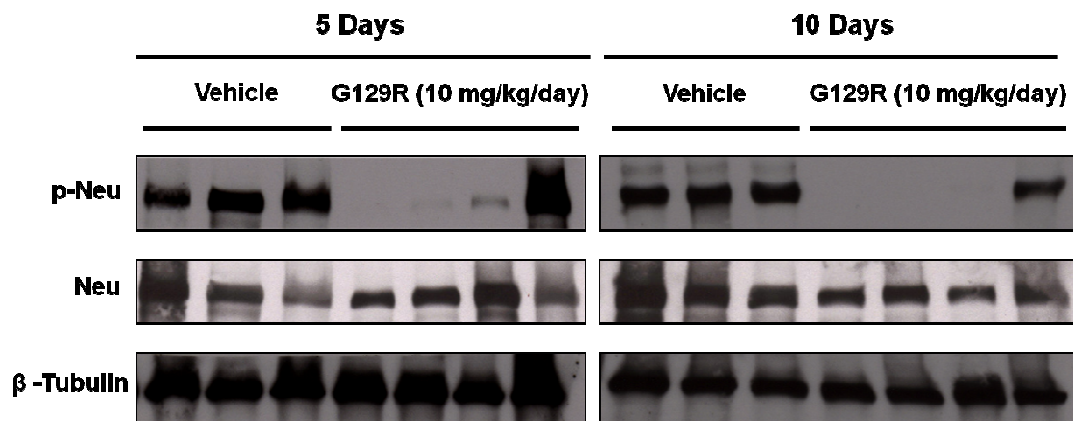
Fig. 4.16   G129R reduced Neu phosphorylation in tumors from randomized MMTV-*neu* mice

Tumor-bearing MMTV-*neu* mice were randomized into Vehicle or G129R (10mg/kg/day) treatment groups. Mice were treated for 5 or 10 days. Approximately 24 hrs after the final injection, tumors were removed and p-Neu was assessed by western blot.

In the Presence of CAFs, G129R Exhibits Synergistic Effect When Combined With G120R

G120R is a human growth hormone antagonist where the glycine at position 120 is substituted by arginine in native 22-kDa human growth hormone. G120R impairs normal dimerization of the growth hormone receptors activated by growth hormone. A study indicated in breast cancer cells, co-treatment with the combination of G120R and G129R inhibited GH-induced STAT5 and JAK2 activation more effectively than either antagonist individually. Therefore, a similar treatment of the combination of G120R and G129R was used in both MCNeuA monocultured cells and MCNeuA cells co-cultured with N202Fb3 CAFs. As expected, cancer cells did not respond to the treatment in monoculture; whereas, the p-HER2 in epithelial cells in co-culture was largely inhibited by the co-treatment (Fig. 4.17).

	MCNeuA + N202Fb3					MCNeuA			
G129R (µg/mL)	0	0	10	5	10	0	10	0	5
G120R (µg/mL)	0	10	0	5	10	0	0	10	5
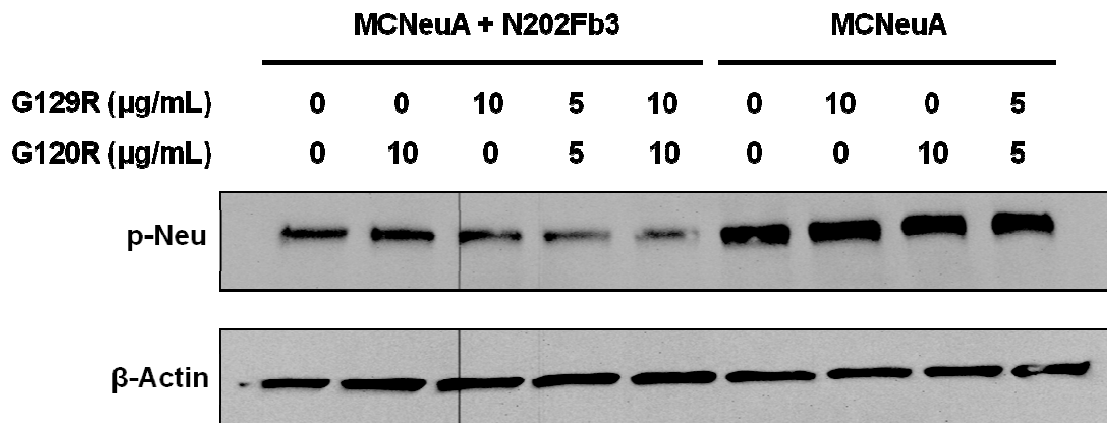
Fig. 4.17   In the presence of CAFs, G129R exhibits a synergistic effect when combined with G120R

Monocultures of MCNeuA cells or co-cultures of MCNeuA cells with N202Fb3 CAFs were treated with G129R (10 µg/ml), G120R (10 µg/ml) or combinations of both. After 24hr, cells were harvested and analyzed by western blot. MCNeuA cells did not respond to treatments in monoculture; whereas, p-Neu was largely inhibited by the co-treatment in co-culture.

Co-culture Model Also Applies to Human Cell Lines

Since the human breast cancer cell line, SK-BR-3, expresses high levels of both HER2 and PRLR, it was selected to examine the validity of the co-culture model with human cell lines. A human CAF cell line derived from an invasive ductal breast carcinoma in a 49 year-old Caucasian female was also selected. Treatment with G129R for 24 hrs had a minimal effect, if any, on p-HER2 when SK-BR-3 cells were grown alone in monolayer. When SK-BR-3 cells were co-cultured at a 4:1 ratio with human CAFs, a reduction in p-HER2 was observed after 24 hrs treatment with G129R (10 µg/ml) (Fig. 4.18A). A similar response was observed when SK-BR-3 cells were co-cultured with mouse N202Fb3 CAFs, indicating that CAFs derived from mice or humans possess the ability to mediate the cross talk with human tumor cells (Fig. 4.18B).
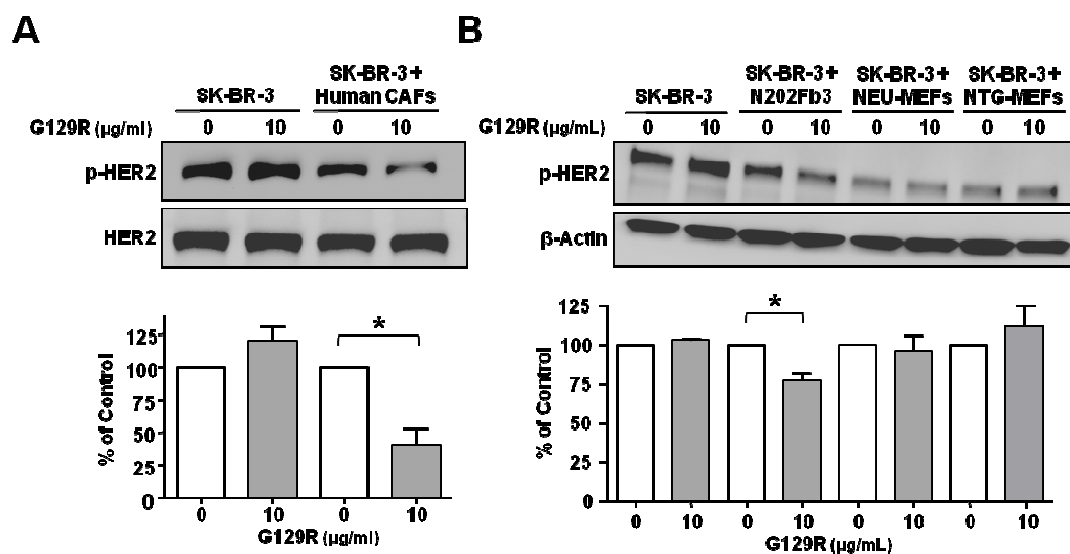
Fig. 4.18   G129R reduces p-HER2 in SK-BR-3 cells when co-cultured with CAFs

(A) SK-BR-3 cells were co-culture at a 4:1 ratio with primary CAFs derived from a human invasive ductal breast carcinoma and treated with G129R for 24 hrs. Western blots for p-HER2 shows that co-culture restores cell response to G129R in SK-BR-3 human breast cancer cells. (B) SK-BR-3 cells were co-cultured at a 4:1 ratio with mouse CAFs (N202Fb3 cells) or primary embryonic fibroblasts (NEU-MEFs and NTG-MEFs) and treated with G129R for 24 hrs. Western blots for p-HER2 indicates that only N202Fb3 cells restored response to G129R in human breast cancer cell lines.

The Mediator of CAFs Interaction With Certain Epithelial Cells May Lie in ECM

In the effort to explore the molecular mechanism behind the interaction between the CAFs and cancer epithelial cells, I already excluded soluble factors selected by CAFs, components on the cell membrane in CAFs and normal fibroblasts derived from embryos. Therefore, my next step was to examine the ECM. The culture was maintained for 4 days before treatments were carried out. In matrigel culture with MCNeuA cells, Neu phosphorylation was clearly suppressed by G129R and up-regulated by PRL both in a dose-dependent manner (Fig.4.19A). In matrigel culture with SK-BR-3 cells, PRL seemed to have minimal stimulatory effect upon p-HER2 (Fig.4.19B). However, G129R still exhibited an evident down-regulation on p-HER2. Interestingly, BT-474 in matrigel failed to respond to any of the treatments (Fig.4.19B).

A

MCNeuA

	PRL				G129R			
(µg/ml)	0	0.1	0.3	1.0	0	1	3	10

p-Neu

β-actin

B

SK-BR-3

	PRL				G129R			
(µg/ml)	0	0.1	0.3	1.0	0	1	3	10

p-HER2

C

BT-474

	PRL				G129R			
(µg/ml)	0	0.1	0.3	1.0	0	1	3	10

p-HER2
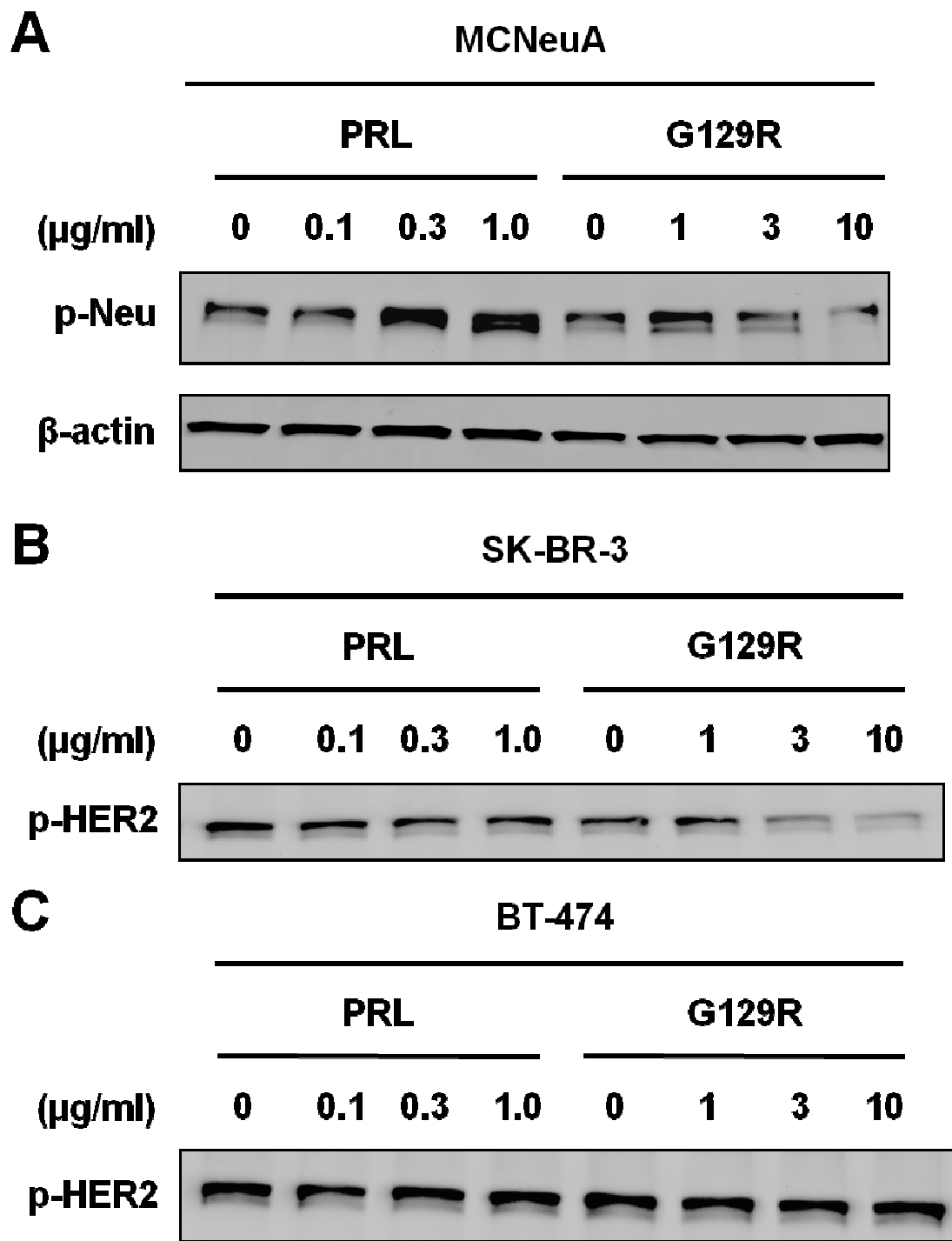
Fig. 4.19   The mediator of CAFs interaction with certain epithelial cells may lie in ECM

Matrigel was used to pre-coat the 12-well plates to form a thin layer at the bottom. MCNeuA, SK-BR-3 and BT-474 cells (1 x 10^5 cells/well) were suspended in the mixture of DMEM (500 µl/well) and EHS (120 µl/well) and placed onto the coated surface. The cell mixture was allowed to settle and attach to the EHS at the bottom before another 500 µl DMEM and 120 µl matrigel were added on top of the gel and the culture was maintained for 4 days before treatments were carried out. Colonies of mouse and human breast cancer cell lines formed in Matrigel were treated with PRL or G129R for 24 hrs. (A) Western blots show that Neu phosphorylation is clearly suppressed by G129R and up-regulated by PRL in a dose-dependent manner in MCNeuA cells. (B) Western blots show that PRL has little stimulatory effect upon p-HER2; whereas, G129R down-regulats p-HER2 in SK-BR-3 cells. (C) Western blots show that BT474 cells do not respond to PRL or G129R treatments.

CHAPTER FIVE

DISCUSSION

HER2 Positive Breast Cancer

It is known that HER2 gene is amplified in approximately 20 to 30% of breast cancers patients [196, 282]. HER2-overexpressing breast cancers are marked with a poor prognosis and fewer successful therapeutic options [122, 258].

The two main signaling pathways in HER2 activation include PI3K-AKT and RAS/MAPK pathway (Fig. 5.1). In RAS/MAPK pathway, after tyrosine receptors are phosphorylated, the adaptor molecules GRB2 and/or SHC bind to the carboxyl tail of the receptor through SH2 domains [83, 223]. The guanine-nucleotide exchange factor, SOS, next interacts with SHC/GRB2 via the GRB2 SH3 domain, and this complex is brought to the receptor at the cytoplasmic surface of the plasma membrane. SOS subsequently catalyzes the dissociation of GDP from RAS, permitting the formation of an activated RAS-GTP complex [185]. RAS-GTP then activates RAF-1, a serine-threonine kinase. RAF-1 in turn phosphorylates and activates MEK, a specific threonine-tyrosine kinase which activates MAPK [173]. In T-47D human breast cancer cells, which express all four members of the class I RTK family, SHC phosphorylation and MAPK activation occur after treatment with NDF. The degree of MAPK activation is markedly reduced, however, when HER2 expression is decreased by single-chain antibody-mediated intracellular retention of the molecule in the endoplasmic reticulum [117].

Another pathway is PI3K/AKT. It is acknowledged that HER2 induces transformation through PI3K/AKT pathway in cooperation with HER3 [8, 133]. Data indicates that HER2 is a favorable heterodimeric partner for HER3. Through this heterodimerization, HER3 provides HER2 the p85 binding motifs to PI3K [231, 260]. This is further supported by that tumors from MMTV-*neu* mice and HER2 overexpressing human breast tumor have activation of PI3K signaling [9, 274, 315]. The activation of PI3K/AKT regulates numerous cellular functions in cancer cells including cell proliferation and survival, cell size and response to nutrient availability, glucose metabolism, epithelial-mesenchymal transition and cell invasiveness, genome stability, and angiogenesis [191, 220, 271, 284].
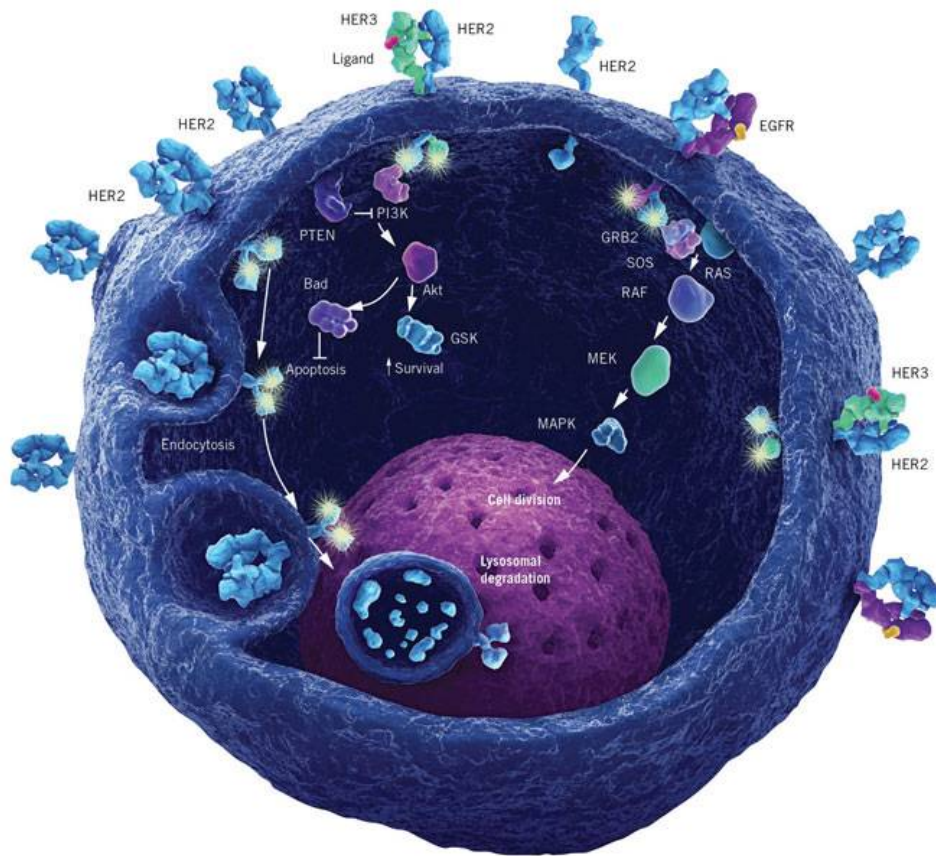
Fig. 5.1   Illustration of HER2 pathway [177]

In RAS/MAPK pathway, after tyrosine receptors are phosphorylated, GRB2 interacts with SOS. SOS subsequently catalyzes the dissociation of GDP from RAS, permitting the formation of an activated RAS-GTP complex. RAS-GTP then activates RAF-1. RAF-1 in turn phosphorylates and activates MEK, which activates MAPK; In PI3K/AKT pathway, the dimerization of HER2/HER3 activates PI3K/AKT which in turn regulates cell survival and apoptosis through GSK and Bad.

To date, there are two drugs designed to target HER2 approved by FDA for the treatment of HER2-positive breast cancers, trastuzumab and lapatinib. Trastuzumab is a humanized monoclonal antibody which is consided as the first targeted therapy for the management of HER2-positive metastatic breast cancers. Trastuzumab has synergistic effect when combined with a variety of chemotherapy drugs. Trastuzumab is also beneficial when combined with endocrine therapy in both ER and HER2-positive patients [156]. Unfortunately, the majority of patients that initially respond to treatment develop resistance resulting in disease progression [285]. The relative refractory state of these HER2-positive breast carcinomas illustrates the need to examine the mechanisms underlying tumor drug resistance and the necessity to examine novel combinations with other agents.

Lapatinib, a small molecule tyrosine kinase inhibitor that blocks the kinase activity of HER1 and HER2 is found to be able to help patients to overcome some trastuzumab resistance [204]. It has been used as a treatment for ER+/EGFR+/HER2+ breast cancer patients (now often called "triple positive") and in patients who have HER2-positive advanced breast cancer that has progressed after previous treatment with other chemotherapeutic agents, such as anthracyline, taxane-derived drugs, or trastuzumab. In clinical study, this drug has been shown to cause remissions in trastuzumab-resistant patients and it may be more effective when given together with trastuzumab [36, 263]. Being stated that, however, HER2-positive breast cancer is still

one of the most difficult subtypes of breast cancer to tackle. Therefore, it is significant to have a better understanding of HER2/Neu signaling pathways through a bigger picture, i.e. not only examing its intrinc molecular events upon various stimuli, but also dissect its responses in the context of tumor microenviroement.

HER2 and PRLR Cross Talk

First of all, HER2 is not the only player in HER2 positive breast cancer. Many clinical studies investigated the association of HER2 overexpression with breast tumor size. In a group of 209 consecutive female patients with invasive operable breast cancer from a defined urban population observed for a median of 30 years, western blots suggested HER2 expression was related to the ductal histologic type, poor histologic grade, and high mitotic count, but not to tumor size, axillary nodal status [273]. This is consistent with my study. In the group of 10 mice with mammary tumors in different sizes, I observed no correlation between tumor size and Neu phosphorylation level. Also p-Neu levels in metastatic tumors in lungs did not appear to be consistent with that in original tumors. In another group of 10 mice with the same tumor onset, no correlation was observed between tumor onset and Neu phosphorylation level.

These results could be potentially explained by the heterogeneity of tumor cells in breast cancer. It is obvious that HER2/Neu is not the only driving force in tumor growth. The presence of other growth factor receptors such as ER, PR or PRLR could also play a role in tumor growth via their own tumorigenic mechanism as well as cross talk with HER2/Neu. Simply, a HER2-positive and PRLR-negative tumor would be less

aggressive than a HER2-positive and PRLR-positive tumor. As a result, the characteristics of breast tumors are determined by the synergistic effect of the overexpression of HER2 and other receptors instead of by the effect of any single factor.

In addition, it was demonstrated that HER2/Neu and PRLR were found to be co-expressed in breast tumors and various breast tumor cell lines. In primary tumor cells isolated from breast tumor derived from female MMTV- *neu* transgenic mice, the FACS analysis indicated that EpCAM was found to be expressed in more than 99% primary tumor cells, nearly 94% of which also expressed PRLR. Also, immunoprecipitation detected PRLR expression in both MCNeuA and N202Fb3 cells derived from breast tumor of a female MMTV-*neu* transgenic mouse, though the expression level was far lower than that in T-47D human breast tumor cells. Additionally, immunohistochemistry showed a high level of PRLR expression in human cell lines that also overexpress HER2, such as SK-BR-3, T-47D, BT-474 and BT-483. Consistently, in an earlier study in our lab, PRLR and HER2 were detected in all four ductal carcinoma cell lines (BT-474, MDA-MB-134, BT-483, T-47D) and two of the six cell lines isolated from pleural effusions (SK-BR-3, MDA-MB-453). Expression of PRLR or HER2 was low or near absent in four of the cell lines isolated from pleural effusions (MCF-7, MDA-MB-468, MDA-MB-436, and MDA-MB-231) [250].

Furthermore, varied effects of phosphorylation dependent cross talk have been noted for PRLR and other growth factor receptors. Sheffield and colleagues demonstrated that PRL caused modest MAPK activation and PRL treatment resulted in

EGFR threonine phosphorylation in the normal murine mammary epithelial cells (NMuMG) model system [91-92, 149, 234]. In the NMuMG cells, PRL decreased both basal and EGF-induced EGFR tyrosine kinase activity. Furthermore, PRL co-treatment dramatically inhibited EGF-induced RAS/MAPK signaling and EGF-induced mitogenesis. In contrast, the Clevenger laboratory demonstrated that several human breast cancer cell lines including T-47D, MCF-7 and MDA-MB-231 exhibited synergistically increased motility when treated with the combination of PRL and EGF compared to the sum of responses to each individual factor [194].

The first study demonstrating the cross talk between HER2 and PRLR was done by a Japanese group in 2000. Yamauchi *et al.* reported that human breast carcinomas overexpressing HER2 have higher proliferative and metastatic activity in the presence of autocrine PRL. They used a neutralizing antibody or dominant negative (DN) strategies or specific inhibitors to show that activation of JAK2 by autocrine secretion of PRL is one of the significant components of constitutive tyrosine phosphorylation of HER2, its association with GRB2 and activation of MAPK in human breast cancer cell lines that overexpress HER2 (Fig. 5.2). Furthermore, the neutralizing anti-PRL antibody or HER2 antisense oligonucleotide or DN-JAK2 or JAK2 inhibitor or DN-RAS or MAPK inhibitor inhibits the proliferation of both untreated and PRL-treated cells. The conclusion was drawn that autocrine secretion of PRL stimulates tyrosine phosphorylation of HER2 by JAK2, provides docking sites for GRB2 and stimulates RAS-MAPK cascade, thereby causing unrestricted cellular proliferation [306]. Similar to what they observed, our previous study examined the phosphorylation status of HER2 and activation of MAPK,

STATs, as well as PI3K-AKT signaling cascades in response to trastuzumab, G129R or a combination of the two in either the absence or presence of exogenous PRL. As a single agent, trastuzumab was more effective than G129R at inhibiting AKT phosphorylation; whereas, G129R was superior at blocking STAT3 and STAT5 activation. G129R was also able to directly inhibit the HER2 phosphorylation. Additionally, the combination of trastuzumab and G129R had an additive inhibitory effect on HER2 and MAPK phosphorylation, confirming that the MAPK signaling is a converging pathway shared by both HER2 and the PRLR. Finally, the combination of trastuzumab and G129R also additively inhibited cell proliferation *in vitro* and *in vivo* as measured by inhibition of the growth of T-47D and BT-474 xenografts in athymic nude mice [250].

In this dissertation, MMTV-*neu* mice bearing spontaneous tumors were used as a model of HER2 breast cancer to further examine the efficacy of G129R. Compared to pretreatment tumor biopsies, G129R decreased p-Neu in a dose- and time-dependent manner after 5 days of treatment and p-Neu was nearly abolished after 10 days of treatment in a majority of the mice. Likewise, p-Neu was reduced in the group of mice randomized to receive G129R (10 mg/kg/day) compared with the group that received the Vehicle. Similar to the *in vivo* studies, but to a lesser extent, G129R (10 μg/ml) was able to reduce p-Neu in cultured tumor chunks; however, it had no effect on monocultures of primary epithelial cells or an epithelial cell line (MCNeuA) derived from a spontaneous mammary tumor of an MMTV-*neu* mouse. These results suggest that cross talk between the PRLR and Neu is only observed when the tumor microenvironment is intact.

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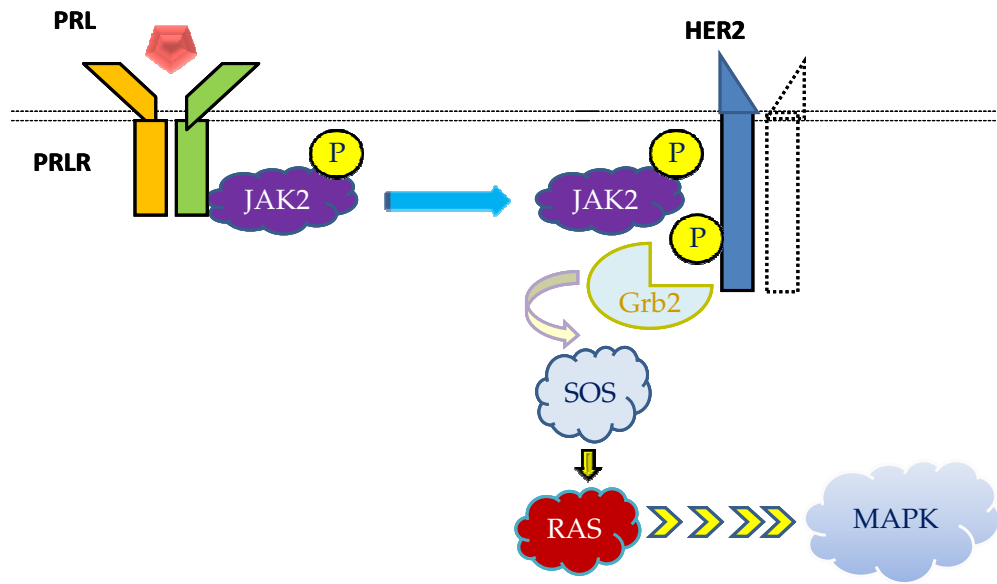
Fig. 5.2   Demonstration of the cross talk between HER2 and PRLR

The activation of Janus kinase (JAK2) by autocrine secretion of PRL is one of the significant components of constitutive tyrosine phosphorylation of HER2. It initiates the association with GRB2/SOS/RAS and activation of MAPK in human breast cancer cells.

Cancer-Associated Fibroblasts and Extracellular Matrix

Tumor microenvironment is mainly consisted of CAFs, immune cells, endothelial cells and the surrounding extracellular matrix secreted primarily by CAFs. Under normal conditions, stroma and epithelia are separated by basement membrane in mammary tissue. However, in the event of carcinogenesis, the abnormally growing tumor epithelial cells break through the basement membrane and invade into the surrounding stroma. This allowed the aberrant activation of the pre-existing communication in the form of soluble factors secreted by both tissues while the direct cell-cell contact between tumor epithelia and stroma is also achieved. According to a number of references, CAFs is one of the key components participating in these carcinogenic events. Therefore, CAFs could paly an important role enhancing the crosstalk between HER2 and PRLR.

In this dissertation, it was demonstrated that the response to G129R and PRL could be restored when MCNeuA cells were co-cultured with N202Fb3 CAFs and that replacement of CAFs with normal NTG-MEFs or NEU-MEFs failed to reduce p-Neu in response to G129R. Considering that MCNeuA cells and N202Fb3 CAFs were derived from the same mammary tumor of a female MMTV-*neu* transgenic mouse, it was clear that adding back CAFs, at least partially, reconstituted the original tumor microenvironment which remains intact in tumor chunks and in the tumors *in vivo*. Similar results were observed using human cells, G129R had no effect upon p-HER2 in SK-BR-3 cells when grown in monoculture or co-culture with normal NTG-MEFs or

NEU-MEFs. Cross talk between the PRLR and HER2 was only observed when SK-BR-3 cells were co-cultured with human CRL-7,236 CAFs or mouse N202Fb3 CAFs.

So what is the molecular mechanism behind this epitheli-stromal interaction? What is so unique in CAFs that mediates the cross talk between PRLR and HER2 while other normal fibroblasts fail?

It has been known that CAFs are perpetually activated fibroblasts at the site of the tumor. These cells are thought to be originated from not only local normal fibroblasts, but also bone marrow-derived cells, malignant epithelial cells, and endothelial cells [39, 152, 312]. CAFs directly stimulate tumor cell proliferation by contributing various growth factors, hormones and cytokines (Fig. 5.3). Classical mitogens for epithelial cancer cells, such as hepatocyte growth factor, epidermal growth factor, fibroblast growth factor, are all vastly expressed by CAFs contacting different tumor types. Beside growth factors, pro-inflammatory cytokines, such as interleukins, interferons and members of the tumor necrosis factor family, are produced both by stromal and cancer cells, and exert tumor-modulating effects, such as SDF-1, CXCL14, CCL7, and IL-6 [236]. This expression by CAFs of cytokines and chemokines leads to immune cell infiltration that in turn promotes angiogenesis and metastasis [106]. On the other hand, CAFs synthesize many of the constituents of the fibrillar ECM such as type I, type III and type V collagen, and fibronectin [238, 275]. They also contribute to the formation of basement membranes by secreting type IV collagen and laminin [48].
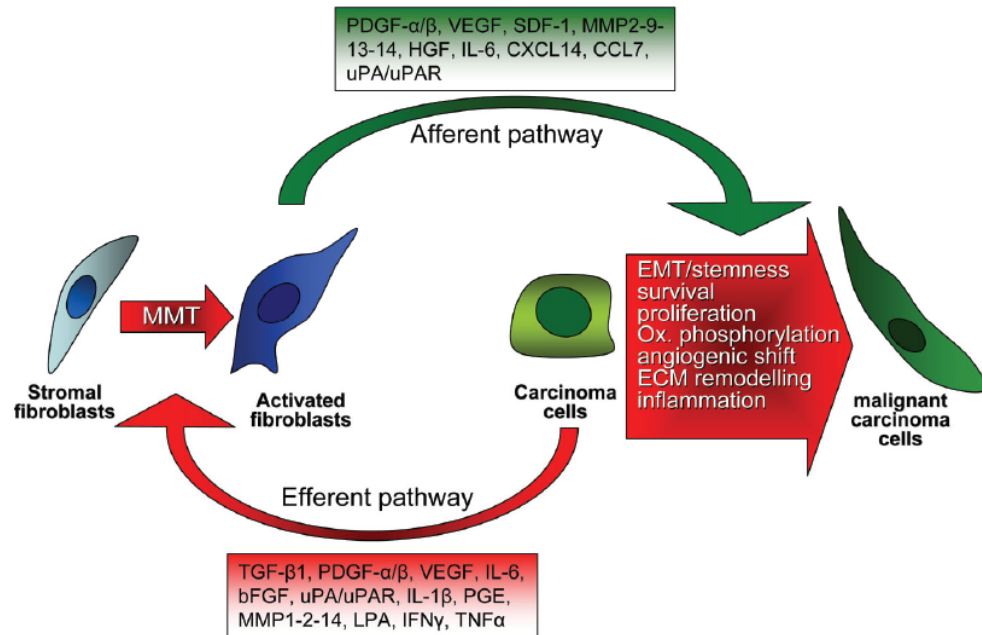
Fig. 5.3   Interplay between CAFs and tumor cells

Tumor progression needs a positive and reciprocal feedback between CAFs and cancer cells. Cancer cells induce and maintain the fibroblasts activated phenotype which, in turn, produce a series of growth factors and cytokines that sustain tumor progression by promoting ECM remodelling, cell proliferation, angiogenesis and EMT [53].

The ECM is composed of an interlocking mesh of fibrous proteins and glycosaminoglycans (Fig. 5.4). The important molecular components include heparin sulfate (HS), collagen, fibronectins and laminins. HS is a linear polysaccharide that binds to a variety of protein ligands and regulates a wide variety of biological activities, including developmental processes, angiogenesis, blood coagulation and tumor metastasis. Collagen is a large molecule composed of a triple helix, which generally consists of two identical chains (α1) and an additional chain that differs slightly in its chemical composition (α2). The common types of collagen are fibrillar collagen (Type I, II, III, V, XI) and basement membrane (Type IV). Fibronectins are proteins that connect cells with collagen fibers in the ECM, allowing cells to move through the ECM. Fibronectins bind collagen and cell surface integrins, causing a reorganization of the cell's cytoskeleton and facilitating cell movement. Laminins form networks of web-like structures that resist tensile forces in the basal lamina. They also assist in cell adhesion. Laminins bind other ECM components such as collagens, nidogens, and entactins [229].

Therefore, there are at least two forms of interaction between epithelium and stroma in cancer. One is through the communication of soluble bioactive factors secreted by CAFs and tumor epithelial cells (Fig. 5.3). The other form is through the direct cell-cell contact. This cell-cell contact is primarily mediated by extracellular matrix produced mainly by CAFs.

To differentiate the effects bridged in the precence of CAFs is solulable factors or insolable, large ECM components produced by CAFs, an experiment using a transwell

system was conducted to physically separate MCNeuA cells from N202Fb3 CAFs, yet still allow soluble secreted molecules to permeate back and forth between the cells. G129R had no effect on p-Neu in the absence of cell–cell and/or cell–matrix contacts, suggesting that the bioactive molecule is not a soluble growth factor. On the other hand, MCNeuA cells cultured with physically disrupted membrane components of N202Fb3 CAFs did not restore cross talk between PRLR and HER2, suggesting that the interaction between tumor epithelia and stroma required CAFs to be alive. Through the process of elimination, I speculate that PRL and G129R modulate the expression of an ECM component by CAFs and that this ECM component enables cross talk between the PRLR and Neu to occur in some epithelial cells.
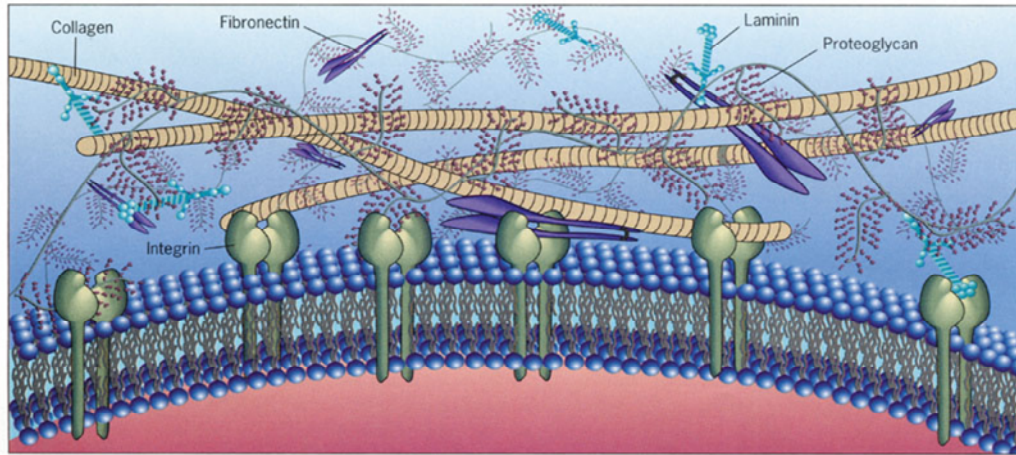
Fig. 5.4   Illustration of extracellular matrix

The ECM is composed of an interlocking mesh of heparin sulfate (HS), collagen, fibronectins and laminins. Integrin, the transmembrane receptor is the main cell surface receptor interacting with the ECM [150].

After excluding soluble factors selected by CAFs, components on the cell membrane in CAFs and normal fibroblasts derived from embryos. The ECM using matrigel was examined. Matrigel is the trade name for a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells and marketed by BD Biosciences. This mixture resembles the complex extracellular environment found in many tissues and is used by cell biologists as a substrate for cell culture. Matrigel, which primarily consists of laminin, collagen IV, heparan sulfate proteoglycans, and enactin, is considered to be a reconstituted basement membrane preparation. The growth factor reduced (GFR) used in the project is a version of matrigel that has been modified to reduce abundance levels of these growth factors including basic fibroblast growth factor, epidermal growth factor, IGF-I, TGF-β, platelet-derived growth factor, and nerve growth factor. The method used to prepare this product effectively reduced the level of a variety of growth factors except for TGF-β which may be bound to collagen IV and/or sequestered in a latent form that partitions with the major components in the purification procedure [139].

In matrigel culture with MCNeuA cells, Neu phosphorylation was suppressed by G129R and up-regulated by PRL both in a dose-dependent manner. In matrigel culture with SK-BR-3 cells, PRL seemed to have minimal stimulatory effect in p-HER2. However, G129R still exhibited an evident down-regulation on p-HER2. Interestingly, BT-474 in matrigel failed to respond to any of the treatments.

Taking all the results together, I believe that an additional mechanism responsible for the cross talk between the PRLR and HER2, especially in MCNeuA cells, should be considered, which is different from the one reported previously using traditional 2D cell cuture system. Fast and sustained stimulation of p-HER2 and p-MAPK has been reported to occur in response to PRL in human breast cancer cell lines in the absence of CAFs, which is dependent upon the kinase activity of JAK2 to recruit the association of GRB2 with HER2; likewise, a reduction in p-HER2 and p-MAPK has been reported to occur in response to anti-PRL and G129R in human breast cancer cell lines in the absence of CAFs [250, 306]. In my experiment, the modulation of p-Neu in MCNeuA cells and to a less extent in SK-BR-3 cells required the presence of CAFs and was not observed in their absence. It should be noted that the means by which p-HER2 was analyzed in SK-BR-3 cells differed between my study and that of Yamauchi *et al*. [306]. They analyzed p-HER2 by immunoprecipitating HER2 and western blotting for total tyrosine phosphorylation; whereas, I blotted for the phosphorylation of the C-terminal tyrosine residue of HER2/Neu that has been reported to serve as docking site for adapter proteins that modulate MAPK activity and to be necessary for Neu-induced transformation and HER2-induced cell migration [22, 79]. Also, in contrast to this rapid modulation of p-HER2 by PRL in human breast cancer cells, the cross talk observed was delayed. Inhibition of p-Neu in response to G129R in co-cultured MCNeuA cells and inhibition of p-HER2 in co-cultured SK-BR-3 cells was not significant until approximately 24 hrs after treatment. Since the cross talk observed between the PRLR and HER2/Neu required CAFs and took longer to observe, it would appear to be via a different mechanism than

the one previously reported to occur in monocultures of human breast cancer cell lines. I speculate that G129R is inhibiting, and PRL is stimulating, the synthesis of a bioactive molecule in ECM by CAFs that modulates p-HER2/Neu. Another possibility is that the delay is due to the process of forming certain structure, i.e. formation of 3D structure or even CAFs specific 2D structure as I noted in my co-culture experiments which compared the different fibrobalsts.

It should be noted that considerable variability was observed in the reduction of p-Neu in response to G129R (~20–50%) even though the ratio of MCNeuA cells to N202Fb3 cells (4:1) and course of incubation with G129R (10 μg/ml) were identical among many of the co-culture experiments. Some of this variability may be attributed to fluctuations in the proliferation rates among the MCNeuA cells and N202Fb3 CAFs, which resulted in morphological changes in the size and shape the nest-like structures formed between the cells, and reflects an alteration in the interactions between the epithelial cells and CAFs. Since G129R reduced p-Neu significantly at a 4:1 ratio but not at an 8:1 ratio, larger nest-like structures formed because of excessive MCNeuA cell proliferation may have reduced the critical interactions with CAFs. A net reduction in p-HER2 was observed when SK-BR-3 cells were incubated with MEFs because of alterations in proliferation. SK-BR-3 cells proliferated more slowly in the presence of MEFs than CAFs resulting in large nests of fibroblasts surrounding small islets of SK-BR-3 cells. The largest variable appears to be due to differences in cell passage numbers, particularly in the CAFs. Many of my late passage N202Fb3 cells lost the ability to modulate p-Neu in MCNeuA cells. I speculate that these variables alter the synthesis and

deposition of an ECM component by the CAFs, necessary for cross talk between the PRLR and Neu.

Possible Cellular and Molecular Mechanism

I believe direct interactions with ECM components in the tumor microenvironment may activate cell adhesion molecules, such as the integrins, and result in the activation of signal transduction pathways [137]. Many literatures showed that integrins were actively involved in carcinogenesis in the form of interacting with ECM components. Meanwhile, integrins were also involved in both PRLR and HER2 activities.

A study has shown that the integrin-mediated adhesion to ECM components modulates the responsiveness of epithelial cells to PRL. Interaction of β1-integrin with the basement membrane component laminin 1 modulates the ability of PRL to stimulate STAT5 DNA-binding activity and express milk proteins, while the interaction of the same integrin with the stromal component collagen I does not [3, 265, 317]. Clevenger et al. demonstrated that following PRL stimulation, a complex between the transmembrane glycoprotein signal regulatory protein-α (SIRPα) and the PRLR, β1-integrin, and JAK2 in estrogen receptor-positive and negative breast cancer cells is formed. Overexpression of SIRPα in the presence of collagen 1 increased PRL-induced gene expression, phosphorylation of JAK2, STAT5, and MAPK, and PRL-stimulated cell growth. This indicated interactions between the PRLR/β1-integrin complex and collagen I modulate the responsiveness of breast cancer cells to PRL and to contribute to breast cancer progression [103]. Furthermore, Giancotti *et al.* reported *ex vivo* studies that indicate

beta 4 forms a complex with Neu and enhances activation of the transcription factors STAT3 and c-Jun. *In vivo*, loss of beta 4 signaling suppresses mammary tumor onset and invasive growth and enhances the efficacy of Neu-targeted therapy. These results indicate that beta 4 integrin promotes tumor progression by amplifying Neu signaling [121].

Putting these all together, therefore, I speculate that the cell and environmental interactions mediated by integrins in tumors may play an important role in inducing cross talk between PRLR and HER2/Neu on certain tumor cells and influence their responsiveness to therapeutic agents such as G129R.

3D structure

Since the matrigel culture system allowed tumor epithelial cells grow into spheres, I can not exclude the possibility that in this assay, the enhancement in the crosstalk between PRLR and HER2 could be induced by the change in cell behavior promoted by 3D cell architecture.

CHAPTER SIX

CONCLUSION

In summary, I demonstrate that CAFs, but not normal fibroblasts, play an important role in modulating the cross talk between PRLR and HER2/Neu. The inhibitory effect of G129R on p-HER2/Neu in tumor epithelial cells depends on, to a certain degree, direct contact with CAFS. My evidence further suggests that the cross talk between the two receptors is likely enhanced by insoluble ECM components produced by CAFs, which probably explained why G129R is more effective in decreasing p-Neu in spontaneous MMTV-*neu* mouse mammary tumors *in vivo* and in cell culture. My result suggests that monoculture models may have poor pre-clinical predictive value for certain drug response. The significance of this finding is that caution must be taken when extrapolating the clinical benefits of drugs using monocultured breast cancer cell lines because of fundamental differences in cell behaviors between monoculture and co-culture systems in the context of tumor microenvironment. The direct co-culture model and *in vivo* studies highlight the significant difference in response to PRL and G129R when compared to monoculture systems, further proving the importance of an accurate model for therapeutic drug evaluation in the preclinical settings.

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