UNIVERSITÉ DE STRASBOURG

ÉCOLE DOCTORALE Sience et Vie de Strasbourg

U 1109

THÈSE présentée par :

Alexia ARPEL

soutenue le : **06 Décembre 2013**

pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Discipline/ Spécialité : Pharmacologie

DEVELOPPEMENT PRECLINIQUE DE PEPTIDES THERAPEUTIQUES TRANSMEMBRANAIRES APPLIQUES AU TRAITEMENT DU CANCER DU SEIN

THÈSE dirigée par :

Monsieur BAGNARD Dominique	MCU, université de Strasbourg
Monsieur LAQUERRIERE Dominique	Professeur, université de Strasbourg

RAPPORTEURS :

Monsieur SCHNEIDER Dirk	Professeur, université de Mainz
Monsieur BENTIRES-ALJ Mohamed	Group Leader, FMI

AUTRES MEMBRES DU JURY :

Madame RIO Marie-Christine	Professeur, université de Strasbourg
Monsieur HUBERT Pierre	CR, Aix-Marseille université

Alexia Arpel

DEVELOPPEMENT PRECLINIQUE DE PEPTIDES THERAPEUTIQUES TRANSMEMBRANAIRES APPLIQUES AU TRAITEMENT DU CANCER DU SEIN

Résumé

Le domaine transmembranaire des récepteurs membranaires est aujourd'hui considéré comme essentiel dans l'activation et la régulation des voies de signalisation sous-jacentes. Ceci est tout particulièrement le cas pour neuropiline-1 et -2 (NRP1/2), et ErbB2, trois récepteurs impliqués dans la croissance tumorale. Notre laboratoire a initialement démontré qu'un peptide ciblant le domaine transmembrane du récepteur NRP1, bloque l'oligomérisation de ce récepteur et provoque ainsi l'inhibition de la prolifération/migration des cellules tumorales et l'angiogenèse *in vivo*. L'objectif principal de ce travail de thèse était d'élargir cette stratégie aux récepteurs membranaires NRP2 et ErbB2, et ce, dans le contexte du cancer du sein. Mes travaux montrent que ces peptides inhibent la pousse tumorale et les métastases associées dans différents modèles de cancer du sein. Les effets anti-tumoraux peuvent s'expliquer par les propriétés anti-angiogéniques et anti-prolifératives des peptides démontrées *in vitro* et *in vivo*. J'ai également disséqué le mécanisme d'action du peptide ErbB2 et montré que le peptide inhibiteur de NRP2 induit des effets secondaires rédhibitoires (promotion des métastases osseuses). Dans l'ensemble, mes recherches valident le potentiel thérapeutique de cette stratégie peptidique et renforce l'idée d'un développement clinique de ces composés. D'une terre inconnue à une terre d'espoir, le cœur de la membrane est incontestablement une nouvelle source d'inspiration pour le développement des médicaments de demain.

Cancer du sein, Peptides transmembranaires, Neuropiline-1, Neuropiline-2, HER2, Métastases, Etude préclinique, Imagerie

Résumé en anglais

The role of transmembrane domains (TMD) in membrane receptor activation and regulation is nowadays appearing as a key step of cell signaling. This has been indeed evaluated for neuropilin-1 and -2 (NRP1/2) and ErbB2 receptors, three membrane receptors whose signaling has clearly been implicated in tumorigenesis. Our team had demonstrated that a synthetic peptide blocking the transmembrane domain of NRP1 blocked NRP1-dependent signaling leading to the inhibition of glioma cell proliferation/migration and tumor associated angiogenesis *in vivo*. The major goal of this thesis project was to extend this novel strategy to NRP2 and ErbB2 in the breast cancer context. Thus, I was able to demonstrate for the first time that the use of peptides, inhibiting the TMD of these receptors, was able to inhibit tumor growth and related metastases *in vivo*, in three different breast cancer mouse models that I have developed in the laboratory. These results were supported by *in vitro* experiments demonstrating anti-proliferative and anti-angiogenic properties of these peptides. Besides, I was able to dissect the mechanism of action of the peptide targeting ErbB2 receptor *in vitro* and *in vivo*, and I provided data excluding NRP2 as a target because of an unexpected promotion of bone metastasis. Altogether, my data offer convincing evidences to further develop MTP-ErbB2 and MTP-NRP1 peptides as novel therapeutic compounds for patients suffering metastatic cancers. From terra incognita to the exploration of a world of hope, the heart of the membrane is becoming a new promising estate for drug design.

Breast cancer, Transmembrane peptides, Neuropilin-1, Neuropilin-2, HER2, Metastasis, Preclinical studies, Imaging

This project would not have been possible without the support of many people.

Many thanks to my PhD jury members for their insightful comments. I would like to first thank Marie-Christine RIO for accepting the role as president of my jury and internal reviewer and for her time evaluating my work. Thanks to Mohamed Bentires-alj and Dirk Schneider, external reviewers, for their time to judge my work. Last but not least, Pierre Hubert, examiner, for the great and very nice technical discussions during my PhD.

I am also deeply thankful to my thesis director Dominique Bagnard, who supervised my thesis. I had a great time working with you and I really appreciated your human and technical qualities in any way. You lead me onto many interesting and challenging paths and you were always available for me, even on Sundays and even when you were overwhelmed with your courses, start up, exams... Thank you for your patience which was really needed time to time to handle me and for being an open person to ideas. I have learned a lot from you. Thank you, for the wonderful time we had together with the Dream B "family" team (wine and cheese, crêpes party, marches gourmandes...)! I could not have imagined having a better advisor and mentor for my PhD!

I would also like to express my thanks to Patrice Laquerrière my thesis co-director for all your contributions during these three years and specially to have helped me the 24th of December with the CT acquisitions of my animals.

Thanks to Gertraud Orend who offered guidance and support during my PhD thesis.

My greatest appreciation and friendship goes to the Dream B "family" team.
Thanks to the lovely dream ladies first.
Thanks to my soul sister Edwige, chatouny. You have learned so much to me! I have spent so abundant super moments with you, we had so much fun together, it was great. I loved the moon walking in the animal house and the body building sessions together!
Also, thanks to you: UR, oritoo, my little sister at the very beginning, than my twin sister and lastly my big sister (Aurore ya plus d'images!! HELP). You are a great person to work with; I like the madness in you and I can always count on you. We have spent so much good moments! I loved the many apéros we shared together (especially when the red wine color would bridge to the beauty spot)!
Thanks to Nur1, justone, you brought a nice and "cool" atmosphere in the lab, thanks for all the help given to me during the thesis defense stressful period.
I would like to thank the Dream boys team.
Bobitto, my twin brother. The one I had on the phone every evening before an oral presentation, asking always the same questions, and you would always answered again and again. Thank you for your great support from the very beginning, during hard moments, and up to the end! It was lovely to work with you day after days.
Lionelitto, also a great member of my Dream B sibship. You were always there to help me at any time of the day, thank you very much for that!

Thank you G.I. JOE, Miku for your investment in the confection of weapons for my thesis film! You are really reliable and it was really nice working with you.
Thank you Gégé, the endless postdoc of our lab. Thanks for all the knowledge given to me, you are best than any library or search engines. Thank you for being a great "grandpa" taking care of all these children around you. Just one thing you should improve is your way to throw brains and red cells.
Last but least Guy! A OUAI! Thank you for being always in a good mood and thank you for all your wise advices in immunology.
Thanks to this wonderful team for which at the beginning their names could not be recalled at first place and now will be in my mind forever! I wish you all the best.

Thanks to Thibaut, Amore mio. I am truly appreciative of all that you have done for me over the years. You are a pillar of strength through all my ups and downs. Thanks for your patience, thanks for being there, thanks for being extremely reliable, thanks for being a great dad. I feel blessed to be part of your life; it is a pleasure to wake up every day by your side.
A few words for my little Jeanne, you're a real sunshine! A new great journey is beginning for the three of us, everything is already fantastic can't wait for the future to come.

And finally, parents, family, extended family, and numerous friends especially the "COM" who make my world. You have endured this long process with me, always offering support and love.

Thanks to all the people who contributed in some way to the work described in this thesis, all the fellow lab mates and persons in my INSERM unit.

Thanks again very much to all of you,

Alexia, alexiittooo, pikpik.

TABLE OF CONTENTS

Abbreviations

aa: amino acid

ADAM: a-dis-integrin and metalloproteinase

ADH: atypical ductal hyperplasia

AIs: aromatase inhibitors

Ala: alanine

ALAT: alanine transaminase

ALP: alkaline phosphatase

AP2: adipocyte protein-2

AREG: amphiregulin

ASAT: aspartate transaminase

ASCO: american society of clinical oncology

Asn: asparagine

Asp: aspartic acid

ATM: ataxia-telangiectasia mutated gene

BACTH: bacterial two-hybrid

Baso: basophil

BRCA1: breast-cancer-susceptibility-gene-type-1

BRCA2: breast-cancer-susceptibility-gene-type-2

BTC: betacellulin

CAM: chick chorio-allantoic membrane

CDH1: cadherin-1

CHEK2: checkpoint-kinase-2-gene

CK18: cytokeratin-18

CK8: cytokeratin-8

CMC: critical micellar concentration

COUP-TFII: chicken ovalbumin upstream promoter transcription factor-2

COX-2: cyclooxygenase-2

CP: core peptide

Creat: creatinine

CT: computed tomography

CUB: complement C1r/C1s, Uegf, Bmp1

CXCR4: chemokine receptor-4

CyA: adenylate cyclase

DA: dimerization arm

DCIS: ductal carcinoma in situ

DMEM: dulbecco's modified Eagle medium

DMPC: di-myristoyl-phosphatidyl-choline

DMSO: dimethyl sulfoxide

DOPC: di-oleoyl-phosphatidyl-choline

DR: dopamine receptor

DSB: double strand breaks

ECD: extracellular domain

ECGS: endothelial cell growth supplement

ECM: extracellular matrix

EDTA: ethylene-diamine-tetra-acetic acid

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

EHS: engelbreth-holm-swarm

EMT: epithelial-to-mesenchymal transition

Eosino: eosinophil

EPG: epigen

EpoR: erythropoietin receptor

ER: estrogen receptor

ER: estrogen receptor

ErbB: epidermal growth factor receptor

ErbB2: epidermal growth factor receptor-2

EREG: epiregulin

ERK: extracellular-signal-regulated-kinase

FACS: fluorescence activated cell sorting

FAK: focal adhesion kinase

FCS: fetal calf serum

FDA: food and drug administration

FGF: fibroblast growth factor

FGFR: fibroblast growth factor receptor

FITC: fluorescein isothiocyanate

FRET: Förster resonance electromagnetic transfer

FSH: follicle stimulating hormone

GBSS: grey's balanced salt solution

Gln: glutamine

Glu: glutamic acid

Gly: glycine

GMP: good manufacturing practice

GnRH: gonadotropin-releasing hormone

GPA: Glycophorin-A

GPCR: G protein coupled receptor

GRAVY: grand average of hydropathy

GTPases: guanosine triphosphatase

HB-EGF: heparin-binding-EGF

HB-FGF: heparin-binding-FGF

HCT: hematocrite

HCV: hepatitis C virus

hEGF: human-EGF

HER2: human epidermal growth factor receptor-2

HGB: hemoglobin

HIV: human immunodeficiency virus

HR: homologous recombination

HSP90: heat shock protein-90

HUVECs: human umbilical vein endothelial cells

I-CliPs: intra-membrane cleaving protease

IDC**:** invasive ductal carcinoma

IGFR: insulin growth factor receptor

IHC: immunohistochemistry

IPHC: institut pluridisciplinaire Hubert Curien

LCIS: lobular carcinoma in situ

LDH: lactate dehydrogenase

LDS: lithium dodecyl sulfate

LH: luteinizing hormone

LTR: long terminal repeat

LVEF: left ventricular ejection fraction

Lympho: lymphocyte

MAM: meprin, A-5 protein, and receptor protein-tyrosine phosphatase mu

MAPK: mitogen-activated protein kinase

MBC: metastatic breast cancer

MCP: major coat protein

MD: molecular modeling

MMP-9: matrix metalloprotease-9

MMTV: mouse mammary tumor virus

Mono: monocyte

MRI: magnetic resonance imaging

MRS: met-related sequence

mTOR: mammalian target of rapamycin

MTP: membrane targeting peptide

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

MUC4: mucin-4

NCCN: national comprehensive cancer network

NCIN: national cancer intelligence network

NMR: nuclear magnetic resonance

NRG: neuregulin

NRP1: neuropilin-1

NRP2: neuropilin-2

PARP: poly-ADP-ribose polymerase

PDGFR: platelet-derived growth factor receptor

PDZ: PSD-95/Dlg/ZO-1

PEA3: polyoma enhancer activator protein 3

PhD: doctor of philosophy

PI3K: phospho-inositide-3-kinase

PK/PD: pharmaco-kinetic/pharmaco-dynamic

PLC: phospholipase-C

PLT: platelet

Plex: plexin

PR: progesterone receptor

Prox-1: prospero homeobox protein-1

PTEN: phosphatase and tensin homologue

PyMT: polyoma middle-T antigen

QPCR: quantitative polymerase chain reaction

RB: retinoblastoma

RECIST: response evaluation criteria in solid tumor

SDF-1: stromal derived factor-1

SEMA: semaphorin

Ser: serine

SERMs: selective estrogen receptor modulators

Sm: small

SP: short peptide

SPECT: single photon emission computed tomography

SSB: single-strand breaks

STAT: signal transducer and activator of transcription

TCR: T-cell receptor

TDLU: terminal ductal lobular units

TEB: terminal end buts

TGFα: transforming growth factor alpha

Thr: threonine

TKI: tyrosine kinase inhibitor

TMD: transmembrane domain

TNBC: triple negative breast cancer

TNFα: tumor necrosis factor alpha

TNM: tumor-node-metastase

Val: valine

VEGF: vascular epithelial growth factor

VEGFR: vascular epithelial growth factor receptor

WHO: world health organization

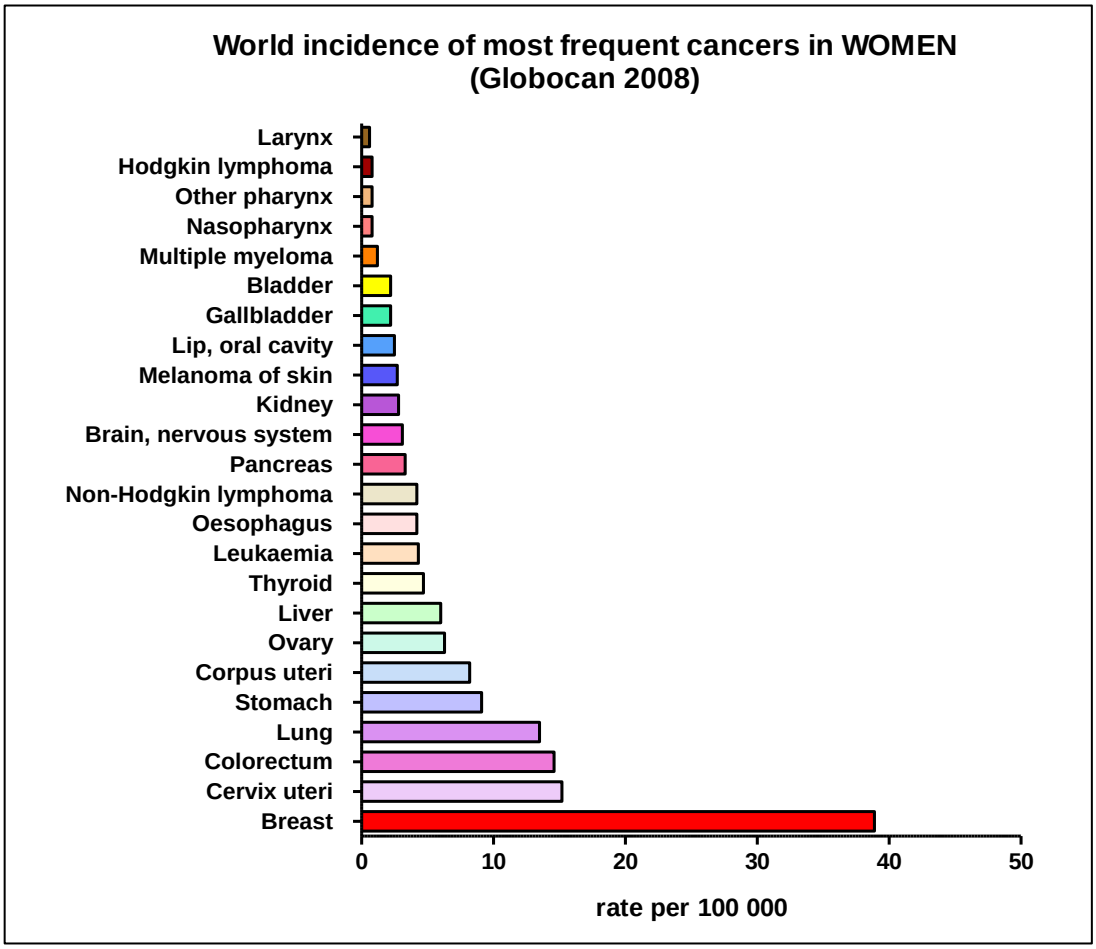


Figure 1: World incidence of most frequent cancers in Women (Globocan 2008)

I) Breast cancer

I.1) Facts and Statistics

Breast cancer is considered to be one of the leading causes of death among women. In France 50 000 women are diagnosed with breast cancer each year (Soria et al., 2012). According to the GLOBOCAN study in 2008 breast cancer has the highest incidence and mortality among most frequent cancers in women (incidence rate of 39 per 100 000 individuals and a mortality rate of 12 per 100 000 individuals) (figure 1). The high incidence and mortality of this disease make breast cancer one of the most relevant health problems in our society. Incidence of breast cancer is closely related with the world region and can reach a fivefold difference. Indeed, lowest incidences are recorded in Asia and in Africa with less than 32 women in 100 000, intermediated incidences are recorded in South America and Eastern Europe with more than 70 women for 100 000 and similar incidences are recorded in Ousters Europe and North America (Nkondjock and Ghadirian, 2005). In France breast cancer incidence has doubled since the 80s, whereas mortality has decreased by 13% (Institut National du cancer, 2011). This can be partially attributed to the national breast cancer diagnosis campaign in France educating women about the risks of breast cancer, and ensuring that women are being screened. Between 2003 and 2010 women participation rate to this campaign increased from 33% to 52.7% (Institut National du cancer, 2011), meaning that widespread screening programs allowed improved diagnosis and coverage of breast cancer with the consequent higher rates of curability. Breast cancer related deaths are mainly due to the incurable nature of metastatic breast cancer. The prognosis for metastatic breast cancer (MBC) is very poor, with an estimated 5 years survival not exceeding 26% (Lu et al., 2009). The probability of death from localized breast cancer (in situ breast cancer) ranges from 0.03 to 0.1 and this probability is considerably worsen for patient with distant disease, 0.70 to 0.85 (Schairer et al., 2004). Since breasts are not essential to sustain life surgery is offering a radical but efficient therapeutic option. Thus breast cancer cells inflict their lethal effect when they metastasize to distance organs: the bone, lungs, liver and brain. For example in bones, the most common organ affected by metastatic breast cancer (MBC), cancer cells will modify the local microenvironment by recruiting and modulating the activity of the host cells. Osteoclasts will be activated resulting in osteolytic lesions which will be

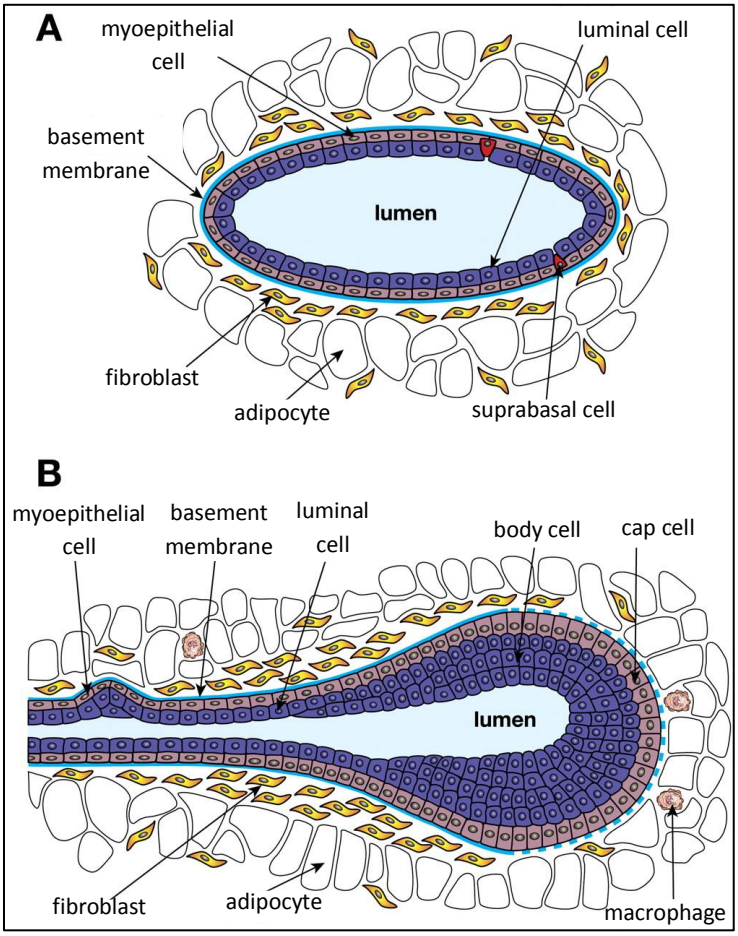


Figure 2: Schematic representation of A) a duct and B) a TEB (Visvader, 2009)

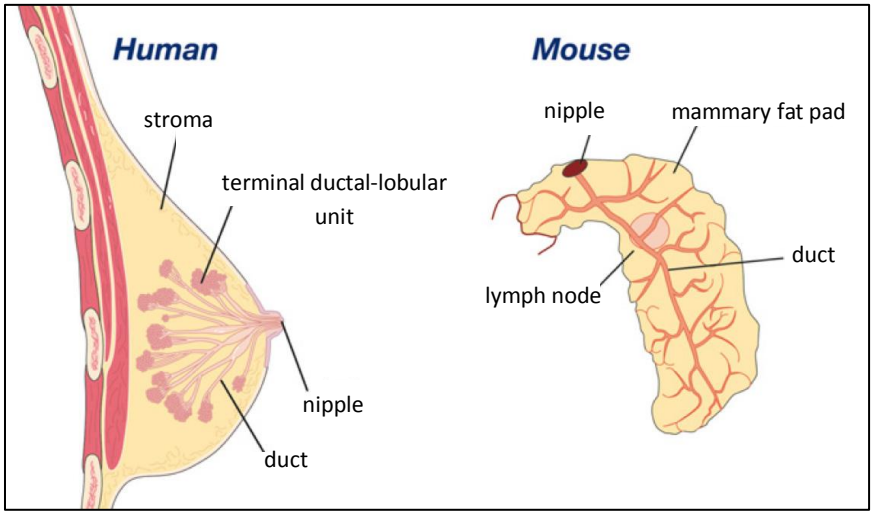


Figure 3: Schematic representation of the human and the mouse mammary glands (Visvader, 2009)

associated with bone pain, pathological fractures and cord compression (Hussein and Komarova, 2011). Hence, the death of woman due to breast cancer remains a sobering fact and indicates the need to develop new strategies and therapeutic tools.

I.2) Anatomy

The breast lies on top of the pectoralis major muscle, which is supported by and attached to the front of the chest wall on either side of the sternum by fibrous strands called Cooper's ligament. The mammary gland is composed of connective tissue, fat tissue and the mammary gland. This latter encloses 15 to 20 lobes further divided into terminal ductal lobular units (TDLU). This functional unit of the breast is formed by the extra-lobular terminal duct and the lobule. Mature mammary ducts and lobules consist of an inner layer of luminal epithelial cells (or luminal cells) and an outer layer of myoepithelial cells wrapped by a basement membrane (figure 2). The epithelial cells produce and secrete the milk, the primary function of the breast. The milk is therefore produced within the lobule and carried to the nipple by the ducts. Each lobe has a collection of ductal lobular units (TDLU) surrounded by connective tissue.

In contrast, mouse mammary gland harbors less connective tissue but more adipocytes, and a clear difference in the ductal tree architecture (figure 3). Nevertheless, numerous evidences points to a striking similarity between species in the cellular organization (Visvader, 2009).

I.3) Mammary gland development

The mammary gland is submitted to continuous multiple changes during lifespan from embryogenesis to pregnancy. Two periods can be discriminated in the mammary growth, one independent of hormone throughout embryogenesis up to puberty and the second is hormone dependent thereafter (Brisken and O'Malley, 2010). During embryogenesis the mammary bud develops from the invagination of the placode into the underlying mesenchyme. The placode is composed of layer of lens-shaped cells which thicken the surface of the ectoderm (Cowin and Wysolmerski, 2010). Then the sprouting of the mammary buds creates a network of branched ducts and forms a rudimentary ductal tree gland. After birth, the mammary development is relatively inert until puberty (Watson and Khaled, 2008). Indeed, the majority of development of the mammary gland occurs through puberty and pregnancy under the guidance of ovarian

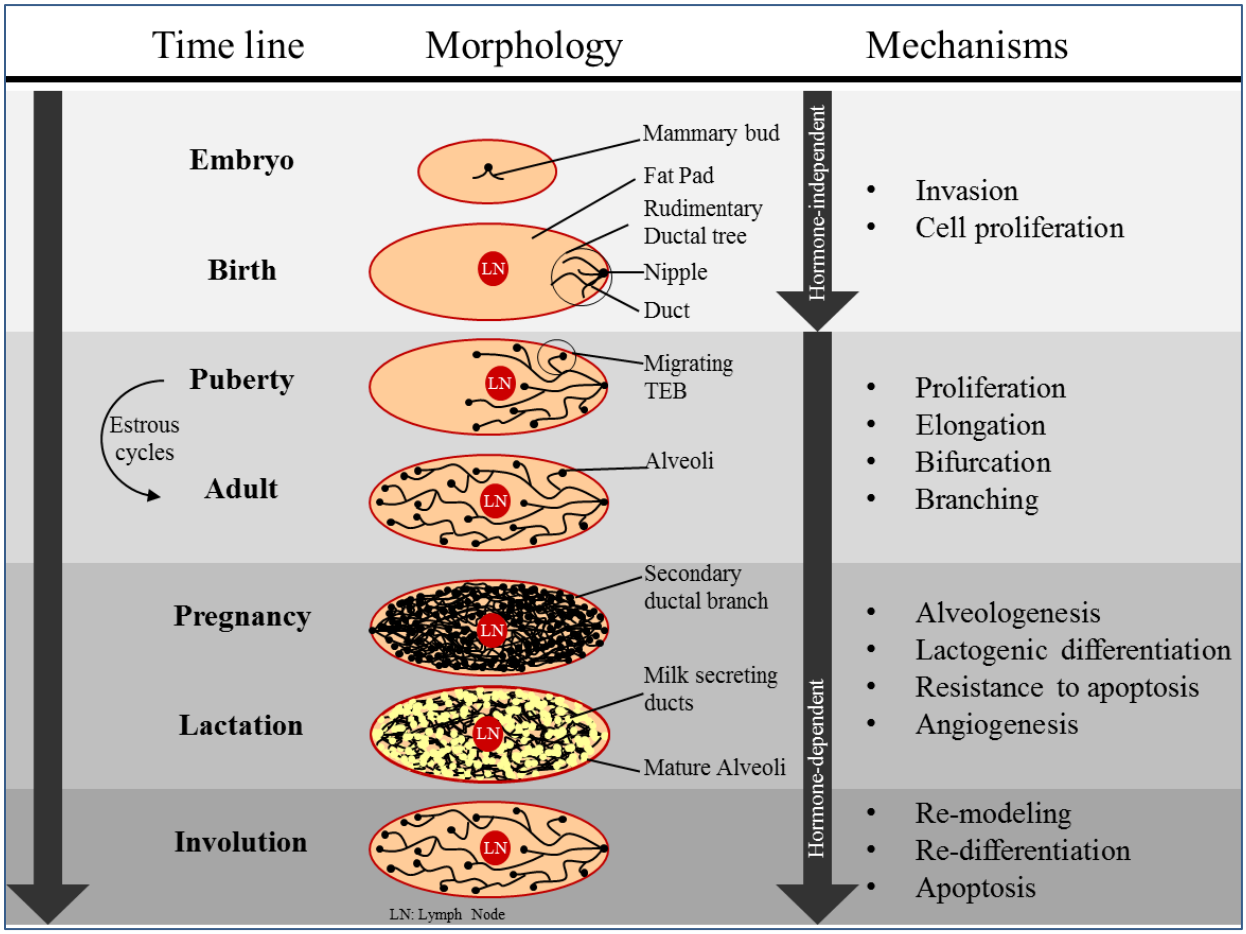


Figure 4: Mammary gland development adapted from (Hennighausen and Robinson, 2005; Fantozzi and Christofori, 2006; Brisken and O'Malley, 2010; Watson and Khaled, 2008)

hormones, growth hormones and growth factors. During puberty under the cyclical production of ovarian estrogen and progesterone, the ducts will elongate and terminal end buts (TEB) will form within the mammary fat (Fantozzi and Christofori, 2006). These TEB appear at the tip of the ducts (figure 4) and will represent the invading front of these, in parallel side branching and proliferation increase importantly (Watson and Khaled, 2008). Following puberty, the adult gland remains relatively quiescent until pregnancy. In the mature gland the entire fat pad is filled with primary and secondary ducts, which develop and involutes within each estrous cycle. During pregnancy under the guidance of progesterone, prolactin and placental lactogens mainly, the mammary epithelium undergoes further development and morphological changes such as lactational differentiation, alveologenesis and sustained proliferation, all of these mechanisms preparing the secretion of milk (Fantozzi and Christofori, 2006; Watson and Khaled, 2008). During this period, blood supply is also increased thanks to the induction of angiogenesis. Moreover, during pregnancy the developing gland is resistant to apoptosis to fight against precipitate involution. After pregnancy and lactation, the loss of sucking stimuli initiates the mammary gland involution into a normal adult gland by apoptosis and re-differentiation (Brisken and O'Malley, 2010; Hynes and Watson, 2010). This causes massive cell death to restore a simple ductal structure again underlying an accurate balance of opposite signaling during the beginning of pregnancy and involution. Remarkably, during the multiple changes of the mammary gland development, mechanisms such as proliferation, invasion, angiogenesis, apoptosis, which are induced in response to hormones, are all involved in breast tumorigenesis.

I.4) Breast cancer risk factors

Most of the known breast cancer risks are listed in table 1. Among them, besides being a female, age is the most important risk factor. Indeed, between 20 and 70 years olds the probability of women to develop invasive breast cancer is multiplied by 16 (American cancer society Breast cancer facts and figures (2011-2012)). About 5 to 10% of breast cancers are hereditary (World Health Organization (WHO)) and among the most common genetic mutations are breast cancer susceptibility gene type 1 and 2 (BRCA1 and BRCA2) increasing the risk of developing breast cancer to 80% (WHO). Less frequent genes mutations include: ATM ataxia-telangiectasia mutated gene (ATM), which encode a protein kinase playing a role in the

Relative Risk	Factor
>4.0	• Age (65+ vs. <65 years, although risk increases across all ages until age 80) • Biopsy-confirmed atypical hyperplasia • Certain inherited genetic mutations for breast cancer (BRCA1 and/or BRCA2) • Mammographically dense breasts • Personal history of breast cancer
2.1-4.0	• High endogenous estrogen or testosterone levels • High bone density (postmenopausal) • High-dose radiation to chest • Two first-degree relatives with breast cancer
1.1-2.0	• Alcohol consumption • Ashkenazi Jewish heritage • Early menarche (<12 years) • Height (tall) • High socioeconomic status • Late age at first full-term pregnancy (>30 years) • Late menopause (>55 years) • Never breastfed a child • No full-term pregnancies • Obesity (postmenopausal)/adult weight gain • One first-degree relative with breast cancer • Personal history of endometrium, ovary, or colon cancer • Recent and long-term use of menopausal hormone therapy containing estrogen and progestin • Recent oral contraceptive use

Table 1: Factors increasing the risk for breast cancer in women (American cancer society Breast cancer facts and figures (2011-2012))

activation of cellular responses to DNA double-strand breaks (Goldgar et al., 2011); TP53, a gene that codes for a tumor suppressor protein (p53) (Børresen-Dale, 2003); CHEK2 or checkpoint kinase 2 gene, coding for an important signal transducer protein of cellular response to DNA damage (Nevanlinna and Bartek, 2006); PTEN or phosphatase and tensin homologue gene, encoding a protein phosphatase that negatively regulates PI3K/Akt signaling, a pathway involved in promoting cell cycle progression and survival (Haiman et al., 2006); CDH1 or cadherin 1 gene, encoding for a protein essential for the formation and maintenance of epithelia, it's suppression results into cellular architecture abnormality and loss of tissue integrity leading to local invasion (Pećina-Šlaus, 2003). Each gene mutation leads in turn to molecular pathogenesis of breast cancer.

Breast cancer risk factors are ranked thanks to relative risk, which is defined by the risk of disease among a population with a specific exposure to the risk among people without that exposure. If the relative risk is below 1.0 there is an inverse relation between the exposure and the disease whereas if the relative risk is higher than 1.0 there is a clear higher risk among the population expose than the unexposed population (table 1).

I.5) The process of tumorigenesis

The concept of cancer is a multistep progression, resulting from the accumulation of mutations. According to Hanahan and Weinberg the process of tumorigenesis is a manifestation of essential alterations switching the cell towards a malignant phenotype which include: sustaining proliferative signaling, evading growth suppression, avoiding immune destruction, enabling replicative immortality, tumor-promoting inflammation, activating invasion and metastasis, inducing angiogenesis, genome instability and mutation, resisting cell death, and deregulating cellular energetics (Hanahan and Weinberg, 2011). For most epithelial cancer, including breast cancer, there is evidence of a traditional linear histologic progression from benign to malignant stages consisting for breast cancer of a progression through normal epithelium to hyperplasia, atypical hyperplasia, pre-invasive carcinoma in situ, to invasive ductal carcinoma and finally metastatic disease. The progression from normal to hyperplasia is a step characterized by an increase in growth of the cells. In atypical hyperplasia in which cells are hyper-proliferative and

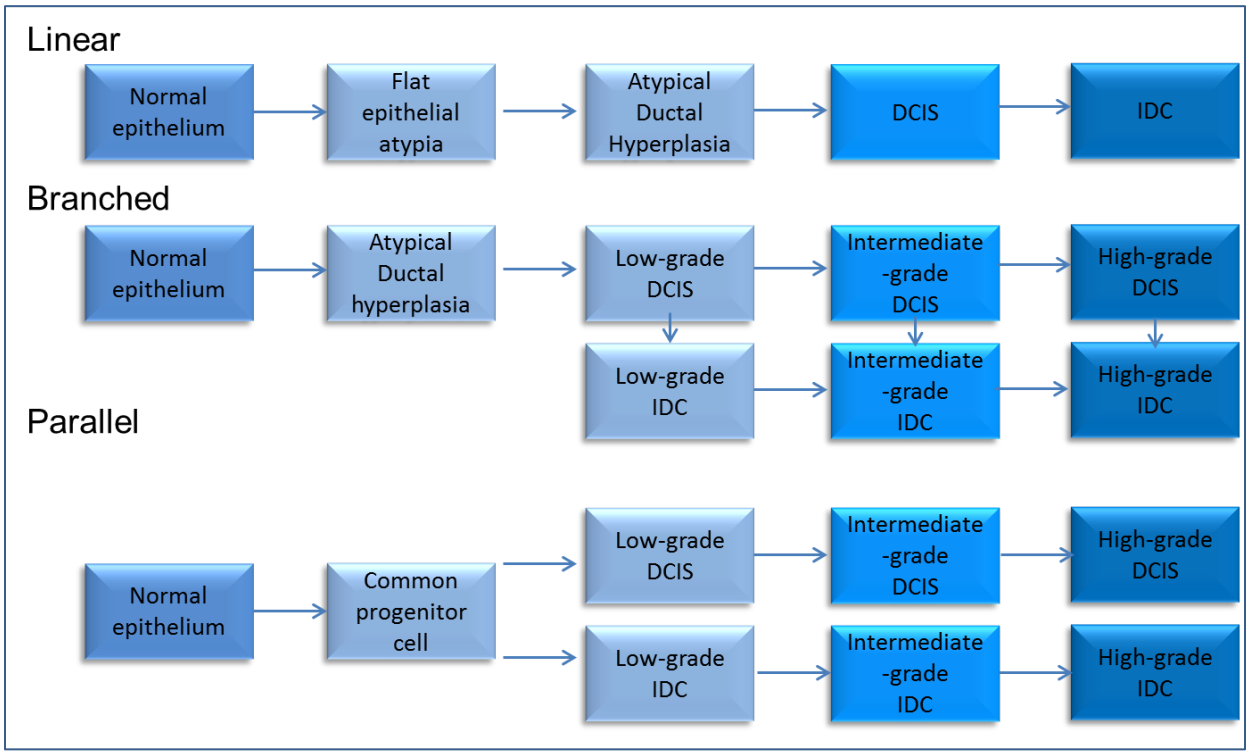


Figure 5: Models of malignant progression of normal breast epithelium to carcinoma (Kaur et al., 2013)

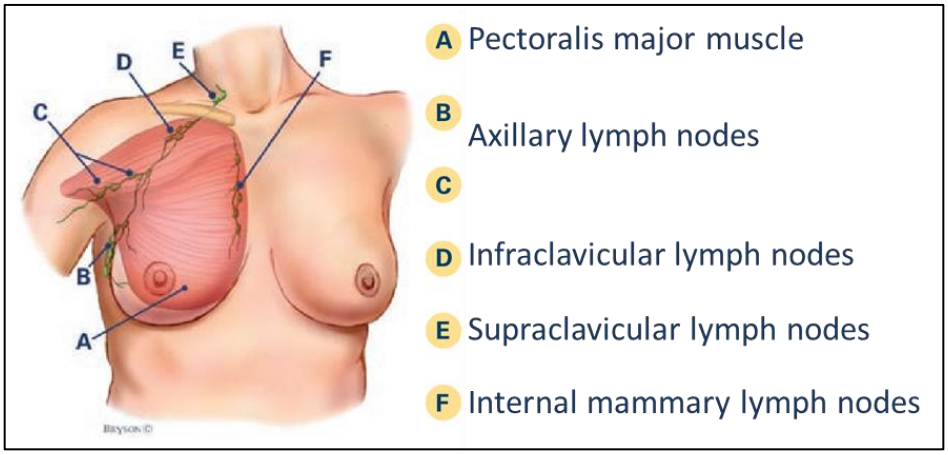


Figure 6: Breast associated lymph nodes (Breastcancer.org Pathology Report Guide, 2013)

the epithelium begins to pile up, abnormal cells can either be found in the breast lobules (atypical lobular hyperplasia) or in the breast ducts (atypical ductal hyperplasia). In the same way pre-invasive carcinoma in situ can be localized rather in the duct or lobules giving rise to ductal carcinoma in situ (DCIS, representing about 80% of all in situ breast cancers) or lobular carcinoma in situ (LCIS, representing the remaining 20% of in situ carcinomas) respectively. Compared to earlier states, in situ carcinomas are described by further growth and the appearance of increased histological and biological diversity. The transition from in situ to invasive carcinoma is defined by the ability of cells to escape from the lobular or ductal compartment. When cells are able to escape from the primary tumorigenic environment and seed into a distant organ the metastatic disease is then taking place (Allred and Medina, 2008). In this classic linear model proposed by Wellings and Jensen (Wellings and Jensen, 1973), in which progression occurs sequentially, the postulates are that: a) DCIS is a direct precursor of invasive ductal carcinoma (IDC) and that b) atypical ductal hyperplasia (ADH) is a direct precursor of DCIS, but the progression of each precursor is non-obligatory. Since then, several models have been proposed to describe the progression from benign to malignant stages based on immunohistological, morphological and epidemiological studies. In the figure 5, beside the linear model, a nonlinear “branched” and “parallel” models are proposed to explain the progression from normal epithelium to invasive breast carcinoma (Kaur et al., 2013). In the branched model DCIS is a progenitor of IDC and can be sub-classified into low grade, intermediate and high grade. Each sub-class of DCIS can be its own precursor or the precursor of the corresponding grades of invasive breast cancer. Whereas, the postulate of the parallel theory is that DCIS and IDC derived from a common progenitor cell and the different grades of DCIS and IDC progress individually and in parallel.

I.6) Diagnosis

A triple diagnosis is required for an accurate and rational examination. It is based on clinical, radiological and pathological criteria (WHO guidelines, National Comprehensive Cancer Network (NCCN) guidelines). In the clinical criteria the diagnosis is based on a systematic inspection and palpation of the breast and the locoregional lymph nodes (axilla, infra- and supraclavicular fossa, see figure 6). A mammography is effectuated in first line for the radiological criteria,

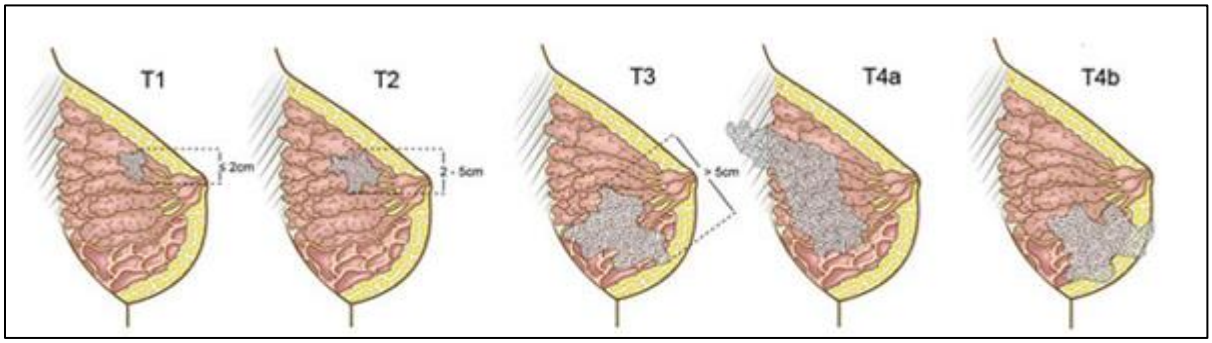


Figure 7: Evolution of tumor breast staging (www.medinfo.ned)

indeed 90% of breast cancers can be detected on mammograms (NCCN guidelines), ultrasounds and magnetic resonance imaging (MRI) can eventually be supplemented. For the pathological diagnosis a core needle biopsy (a very common approach for which large testing sample is available) or a fine needle aspiration (removal of small cell amounts) is manually effectuated.

I.7) Classification of breast cancer

Breast cancer is a very heterogeneous disease and classification is useful for treatment and prognosis. Several classifications exist using different criteria and include histological type, tumor grade, tumor stages and molecular classification.

I.7.1) Histological classification

This classification is based on the morphological characteristics, architectural features and growth patterns of the tumor cells when using a microscope. Histological breast cancers types are roughly categorized into in situ and invasive carcinoma. The identification of the localization of the tumor further discriminates in situ carcinoma into lobular (LCIS, lobular carcinoma in situ) and ductal (DCIS, ductal carcinoma in situ). While lobular carcinoma in situ has low histological variation ductal carcinoma in situ is further subdivided into comedo, cribiform, micropapilary, papillary and solid type. Interestingly, in the case of mice, comedo and cribiform sub-types were observed in the MMTV-NeuNT mouse model of breast cancer used for *in vivo* studies. Comedo ductal carcinoma in situ is composed from the center to the duct wall of cell debris (from necrosis), dying cells and living cells lying on the basement membrane. Cribiform sub-type in situ carcinoma exhibits a lumen in the center of the duct, which is surrounded by cancer cells in which small holes are founded. Invasive carcinoma is also subdivided, this breast cancer type include six subgroups comprising ductal and lobular invasive carcinoma here again based on the localization of the tumor within the mammary gland (Malhotra et al., 2010).

I.7.2) Tumor grade

As histological classification, the breast cancer grading refers to the characteristics that tumor cells exhibit under a microscope. The Bloom-Richardson system is used for grading breast cancers. Here the notion of differentiation is highly important. Indeed, there are 3 degrees of severity of differentiation status of the tumor cells; the grade 1 or G1: the cells are well

Stage	T (Tumor)	N (Lymph Node)	M (Metastases)
x	**Tx**: Primary tumor cannot be assessed	**cNx**: Regional lymph node cannot be assessed	**Mx**: Metastases cannot be assessed
0	**T0**: No evidence of primary tumor	**cN0 / pN0**: No regional lymph node metastasis	**M0**: No apparent metastases
is	**Tis**: Carcinoma **i**n **s**itu: ductal (DCIS) or lobular (LCIS) carcinoma, or Paget's disease of the nipple with no tumor		**M0**: No apparent metastases
1	**T1**: Tumor ≤ 2 cm **mic** ≤ 0.1 cm, **a**. 0.1 < x ≤ 0.5 cm, **b**. 0.5 < x ≤ 1 cm, **c**. 1 < x ≤ 2 cm	**cN1**: Metastasis to movable axillary lymph node **pN1:** Tumor growth to 1 to 3 axillary lymph nodes **pN1mi:** Lymph node tumor ≤ 2.0 mm	**M0**: No apparent metastases
2	**T2**: Tumor 2 cm < x ≤ 5 cm	**cN2**: **a**. Metastasis to axillary lymph node fixed to one another or matted to other structures **b**. Clinically-apparent internal mammary lymph node in the absence of clinically-evident axillary lymph node metastasis **pN2:** Tumor growth to 4 to 9 axillary lymph nodes	**M0**: No apparent metastases
3	**T3**: Tumor < 5 cm	**cN3**: **a**. Metastasis to infra-clavicular lymph node **b**. Clinically-apparent internal mammary lymph node in the presence of clinically-evident axillary lymph node metastasis **c**. Metastasis to supraclavicular lymph node with or without axillary or internal mammary lymph node involvement **pN3:** tumor growth to 10 or more axillary lymph nodes or lymph nodes in other areas around the breast	**M0**: No apparent metastases
4	**T4**: Tumor of any size with direct extension to the chest wall or skin **a**. Extension to chest wall not including pectoral muscle **b**. Oedema, including "peau d'orange", ulceration of the skin or satellite skin nodules confined to the same breast **c**. Both a and b, **d**. Inflammatory carcinoma.		**M1**: Distant metastases

Table 2: TNM classification. "c" for clinical stage, "p" for pathologic state (WHO guidelines; NCCN guidelines; Soria et al., 2012)

differentiated (low grade), the grade 2 or G2: the cells are moderately differentiated (intermediate grade) and the grade 3 or G3: the cells are poorly differentiated (high grade). Outcome of the disease is therefore correlated to the grade (Bloom and Richardson, 1957).

I.7.3) Tumor stage (TNM)

The tumor-node-metastases (TNM) staging classify tumor within 5 stages. This staging offers information about the extent of the disease in distant organs and will also lead the treatment. Remarkably no unique staging system exists, for lymph node staging two sets of category are described (NCCN guidelines), the clinical staging marked with the letter “c”, and the pathologic stage marked with the letter “p” (the later based on the number of axillary lymph node colonized by the cancer cells). Representative images of tumor stages from T1 to T4b are illustrated in figure 7. Stage 0 describes non-invasive breast cancers as in ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). Breast cancers are labeled *Tis* as described in table 2 and do not produce metastasis to lymph node nor distant sites. Stage I designates invasive breast cancer which is divided in two subcategories: Ia includes small cancer, the tumor is no more than 2 cm and has not spread to lymph nodes; Ib the tumor is present or not (in any case size is less than 2 cm), very small breast cancer cells are found in the axillary lymph nodes (≤ 2.0 mm). In both categories cancer cells have not metastasized to distant organs. Stage II is divided into subgroups known as IIa and IIb. Tumor size is not above 5 cm and tumor cells may or may not have spread to axillary lymph nodes. Stage III is divided into three groups IIIa, IIIb and IIIc. No evidence of tumor can be described or tumor size is not above 5 cm but tumor cells have spread at least to 1 axillary lymph node. In Stage IV breast cancer cells have metastasized to distant sites. Then, information from each category allows a stage grouping, which is presented in the table 3.

I.7.4) Receptor status

Breast cancer can also be classified based on immunohistochemistry profile of the presence or the absence of estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor 2 (ErbB2) expression. The diverse groups are composed of the three receptors giving rise to eight subtypes, the composition of the subtype provides both a therapeutic and a

Stage	T	N	M
0	Tis	N0	M0
Ia	T1	N0	M0
Ib	T0 T1	N1mi N1mi	M0 M0
IIa	T0 T1 T2	N1 N1 N0	M0 M0 M0
IIb	T2 T3	N1 N0	M0 M0
IIIa	T0 T1 T2 T3 T3	N2 N2 N2 N1 N2	M0 M0 M0 M0 M0
IIIb	T4 T4 T4	N0 N1 N2	M0 M0 M0
IIIc	Any T	N3	M0
IV	Any T	Any N	M1

Table 3: Stage grouping of breast cancer (WHO guidelines; NCCN guidelines)

prognostic information, moreover there are considerable differences in survival and tumor characteristics among the eight subtypes (Onitilo et al., 2009; Parise et al., 2009).

I.7.5) Molecular classification

Based on cDNA microarrays analysis, six molecular breast cancer subtypes have been determined, with the most important determinants of these subtypes being the presence or absence of expression of the estrogen receptor (ER) or the progesterone receptor (PR), or the amplification/overexpression of the ErbB2 locus. Nevertheless, the number of clearly different molecular phenotypes observed among the breast tumors is not, so far, clearly established, suggesting that we are far from having a complete picture of the diversity of breast tumors. Molecular classification is still a model in development that requires improvement and standardization (Perou et al., 2000; Raica et al., 2009; Visvader, 2009; Eroles et al., 2012; Prat and Perou, 2011).

I.7.5.1) Normal-like subtype

As their name implies, normal-like subtypes have similar expression pattern to normal breast tissue (as adipose tissue, basal epithelial cells and a few epithelial characteristics). The significances of this subtype has still to be clearly established (Peddi et al., 2011).

I.7.5.2) Luminal A and Luminal B subtype

Luminal breast cancers are characterized by luminal epithelium genes, proteins such as cytokeratin 8 (CK8) and cytokeratin 18 (CK18) are spotted. Luminal tumors express ER and/or PR receptor and are HER2 negative. Within the luminal cluster there are at least two subtypes, luminal A and luminal B. Luminal A subtype is less proliferative (low protein expression of Ki67) than Luminal B subtype. Luminal A subtype has also a better clinical outcome compared to luminal B subtype. Indeed luminal B type breast cancers are more aggressive and are usually of high tumor grade (Raica et al., 2009).

I.7.5.3) Her2 subtype

Here the expression of CK8/CK18 is heterogeneous and moderate; this subtype is frequently associated with DCIS. The tumors are characterized by high expression levels of ErbB2 and/or

Cancer stage and type	Primary treatment	Node evaluation	Adjuvant therapy		
			Hormone receptor negative	Hormone receptor positive	ERBB2 overexpression
Stage 0: in situ Lobular carcinoma in situ	No treatment or consider prophylaxis with tamoxifen	–	–	–	–
Ductal carcinoma in situ	Breast-conserving surgery	–	–	–	–
Stage I and II: early-stage invasive	Breast-conserving surgery and radiation therapy	Sentinel lymph node biopsy or axillary lymph node dissection	Chemotherapy	Chemotherapy and endocrine therapy	Chemotherapy and trastuzumab
Stage III: locally advanced Noninflammatory	Induction chemotherapy, followed by breast-conserving surgery and radiation tharapy	Sentinel lymph node biopsy or axillary lymph node dissection	Induction chemotherapy	Induction chemotherapy and postoperative endocrine therapy	Induction chemotherapy and postoperative trastuzumab
Inflammatory	Induction chemotherapy, followed by mastectomy and radiation tharapy	Axillary lymph node dissection			
Stage IV: metastatic Initial or recurrent	Address patient's treatment goals; radiation therapy or bisphosphonates for bone pain	–	Chemotherapy	Endocrine therapy with or without chemotherapy	Trastuzumab with or without chemoptherapy
Recurrent Local after breast-conserving surgery	Mastectomy	Axillary lymph node dissection	Chemotherapy	Chemotherapy and endocrine therapy	Chemotherapy and trastuzumab
Local after mastectomy	Wide excision	Axillary lymph node dissection			
Local inoperable	Induction chemotherapy	Axillary lymph node dissection			

Table 4: Therapeutic options for breast cancer by stage (Maughan et al., 2010b)

overexpression of genes associated with the ErbB2 pathway. In addition, HER2 subtype present usually low ER expression, they are more likely to be of high grade and are poorly differentiated, hence leading to a worse prognosis (Raica et al., 2009; Brenton et al., 2005).

I.7.5.4) Claudin-low subtype

This subtype is characterized by the low gene expression of luminal markers and low expression of tight junction proteins such as claudin 3, 4 and 7, and E-cadherin, a calcium-dependent cell-cell adhesion glycoprotein. On the other hand they present high expression of epithelial-to-mesenchymal transition (EMT) markers, and cancer stem-cell-like features. The majority of claudin-low subtype are triple negative tumors and are of poor prognosis as well (Prat et al., 2010; Peddi et al., 2011).

I.7.5.5) Basal-like subtype

Such as normal-like subtype, basal subtype was so named because the expression pattern mimics that of basal epithelial cells. They are indeed characterized by the expression of CK5/6 and/or EGFR. This subtype is commonly associated with triple negative breast cancer (TNBC) therefore not expressing estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), they also show a high rate of p53 and BRCA1 mutation. These tumors are aggressive and present higher likelihood of being of high grade with poor prognosis (Raica et al., 2009; Brenton et al., 2005; Peddi et al., 2011).

I.8) Treatment

Once breast cancer is diagnosed, the treatment is impossible to generalize because there are so many different sets of circumstances. Treatment decision takes into account different factors such as the age of the patient (more or less than 35), the type and stage of the breast tumor, menopause and the presence or not of hormone receptors. Treatments used for breast cancer include; surgery, chemotherapy, radiotherapy, hormonotherapy and targeted therapy. These latter either being neoadjuvant and/or adjuvant (usually primary therapy is surgery) (NCCN guidelines, WHO guidelines, Soria et al., 2012). Treatment decision can be based on stages of breast cancer and the different therapeutic options are presented in table 4.

Localized tumor*
Negative surgical margins
No diffuse (inflammatory) or multicentric cancer
No malignant-appearing mammographic abnormality after surgery
No previous radiation therapy to the breast or chest wall (precludes further radiation therapy)
*—Large tumor size in small breast may preclude the benefit of breast-conserving surgery (relative contraindication).

Table 5: Qualifying factors for consideration of breast cancer conserving surgery in the treatment of breast cancer (Maughan et al., 2010a)

I.8.1) Surgery

Most women with breast cancer will require surgery (> 80% of women diagnose with breast cancer according to the National Cancer Intelligence Network (NCIN) guide), this is generally the primary therapy. There are two well established surgical procedures for breast cancer; breast conserving surgery and mastectomy.

Pre-operative assessment of the size and extent of the tumor is crucial for determining whether breast conservation surgery is an alternative to mastectomy. Importantly the patient choice is also essential, qualifying consideration of breast cancer conserving surgery are presented in the table 5. In a lumpectomy the surgeons removes only a lump of the breast containing the cancerous area and surrounding margin of the normal tissues. In a partial mastectomy, the area of tissue removed is greater than in a lumpectomy but less than in a quadrentectomy, in which one-fourth of the breast is removed. In both cases the cancerous tissue and the surrounding margin of normal tissues is removed. The conservative surgery has the aim of preserving a normal breast appearance after surgery.

When a very large part or the entire breast is removed the surgery is called mastectomy. Depending on the tissues removed one can distinguish four different types of mastectomy. In simple mastectomy the surgeon removes the whole breast including the nipple without removing lymph nodes or the pectoral muscle. Modified radical mastectomy involves the removal of all the breast tissue with the nipple and lymph nodes in the armpit, here again no muscle is removed from the beneath the breast. In radical mastectomy the surgeon removes the entire breast with lymph nodes and the chest wall muscles under the breast, resulting in the most extensive type of mastectomy. Finally in the subcutaneous mastectomy, while the entire breast tissue is removed the nipple is left intact.

I.8.2) Chemotherapy

Chemotherapy plays an important role in treatment and improves patient outcome (Swain, 2011). It is given in cycles of treatment, which vary in length according to the drug. Chemotherapy can be given alone mainly in case of hormone-negative breast cancers and HER2

Name	Manufacturer	Development stage	Route	Toxicities
Cabazitaxel (Jevtana®)	Sanofi	Phase II	IV	Neutropenia, leukopenia, anemia, febrile neutropenia, diarrhea, fatigue, asthenia
Nab-paclitaxel (Abraxane®)	Abraxis bioscience/ Celgene	FDA-approved (2005)	IV	Neutropenia, ↑GGT, peripheral neuropathy, weakness
DJ-927 (Tesetaxel®)	Genta	Phase II	PO	Neutropenia, sepsis, diarrhea, lethargy
EndoTAG + paclitaxel (EndoTAG®-1)	MediGene	Phase II	IV	Neutropenia, chills and fever
XRP9881 (Larotaxel®)	Sanofi	Phase II	IV	Neutropenia, neuropathy, diarrhea
Polymeric-micellar paclitaxel (Genexol-PM®)	Samyang Genex Co	Phase II	IV	Neutropenia, neuropathy, arthralgia, hypersensitivity

Table 6: Novel taxane formulation in breast cancer (Yared and Tkaczuk, 2012)

negative breast cancers. It is usually preceding or complementary to hormone or targeted therapies when the tumor harbors ER, PR and/or HER2 receptor (Turner and Jones, 2008; Soria et al., 2012). Neoadjuvant chemotherapy is usually given to patient with locally advanced breast cancer downsizing the local tumor and therefore facilitating breast-conserving surgery. Most commons chemotherapies include taxanes and anthracyclines.

I.8.2.1) Taxanes

Taxanes are usually given when lymph nodes are colonized (Soria et al., 2012), these drugs are antineoplastic agents that stabilize microtubule by binding to the interior surface of the beta-tubulin subunit of microtubule, leading to a cell-cycle arrest, altered mitosis and in turn induce cellular death (Oakman et al., 2009; Yared and Tkaczuk, 2012). Docetaxel (Taxotere®) and Paclitaxel (Taxol®) are the most commonly used drugs but recently novel second-generation taxanes have been discovered (Yared and Tkaczuk, 2012) (table 6). Typical course of treatment is presented in the table 8.

I.8.2.2) Anthracyclines

This antibiotics drug class is very common and is the standard adjuvant chemotherapy for breast cancer (Turner and Jones, 2008; Soria et al., 2012), Doxorubicin (Adriamycin) and Epirubicin (Ellence) are the most common anthracycline in clinical practice (Robson and Verma, 2009). Anthracyclines have multiple mechanism of action: 1) they form complexes with DNA by intercalating between base pairs; 2) they inhibit topoisomerase II (Moreno-Aspitia and Perez, 2009; Minotti et al., 2004); 3) they bind to DNA and induce alkylation; 4) they generate free radicals leading to DNA damage or lipid peroxidation; all these mechanisms impair DNA replication and repair, thereby leading to apoptosis. The usefulness of anthracyclines are limited by cardiotoxicity (Petit, 2004). Novel anthracycline formulation include tumor targeted formulation consisting for example of liposomal and pegilated formulation and analog molecules. Typical course of treatment is presented in table 8.

Numerous other chemotherapy drugs can also be used for treating breast cancer. For example, alkylating agents induce intra and inter DNA strand biding, damaging DNA, which in turn abrogates cell cycle and induce cell death. The most common DNA damaging agents are

cyclophosphamide and platinum derivatives such as Carboplatin and Cisplatin (Oakman et al., 2009). Anti-folates inhibit several enzymes in the synthesis pathway of purines and pyrimidines (Chu and Lu, 2008a). Taxoids are a class of drug derivatives from taxol, for example larotaxel (XRP9881) harbors an activity against taxane-resistant breast cancer (Chu and Lu, 2008a).

I.8.3) Endocrine

As mentioned above breast cancer can be classified according to hormone status. Endocrine therapy is used in ER positive breast cancer patients, as these breast cancers are reliant on estrogen for growth. From puberty, the ovaries produce estrogen but after menopause estrogen are converted from androgen by aromatase. Endocrine treatments prevent either the production of estrogen from ovaries or block estrogen actions. There are three classes of hormonal treatment: aromatase inhibitors, gonadotropin-releasing hormone agonists and selective estrogen receptor modulators (Endocrine Treatments for Breast Cancer; Maughan et al., 2010a).

Aromatase inhibitors (AIs), as named, inhibit de production of estrogens from androgens blocking aromatase. They are used as first line adjuvant therapy for ER positive breast cancers in postmenopausal women. Common AIs in the treatment of breast cancer include Anastrozole (Arimidex), Exemestrane (Aromasin) and Letrozole (Femara), their typical course of treatment is indicated in the table 8 (Maughan et al., 2010b; Chumsri et al., 2011).

Gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus. In response to GnRH the pituitary gland produce luteinizing hormone (LH) and follicle stimulating hormone (FSH), two hormones regulating the production of estrogens by the ovaries. Importantly LH secretion from the pituitary gland requires a pulsatile secretion of GnRH, and GnRH agonist mechanism of action through constant infusion of GnRH causes a down regulation of GnRH receptor concentration. Basically, GnRH regulates its own production in the hypothalamus. The pituitary gland is then desensitized, ovaries do not receive gonadotropin stimulation and estrogen is not produced in turn (Magon, 2011). Goserelin (Zoladex) is a common GnRH agonist used in breast

cancer and is given subcutaneously (Maughan et al., 2010b) due to molecule destruction if administrated orally.

Selective estrogen receptor modulators (SERMs) are the reference therapy for premenopausal women. These molecules bind estrogen receptor and alter receptor conformation inhibiting the proliferative effects of estrogens that are mediated through the estrogen receptor. Tamoxifen (Nolvadex) is the most common and successfully SERM used in the treatment of ER positive breast cancers, Tamoxifen is cytostatic and induces a G1 cell cycle block (Osborne et al., 2000). Endocrine mechanism of resistance includes cell switching from ER positive to ER negative, acquired mutation resulting in dysfunctional forms of ER and overexpression of cyclin D1.

I.8.4) Targeted therapy

I will review here the most recent molecular targets in breast cancer targeted therapy. These therapies fight against three hallmarks of breast cancer detailed above, the inhibition of proliferative signaling (targets include here ErbB family and signaling), interfering with DNA repair pathways (BRCA1, BRCA2) and anti-angiogenic therapy.

I.8.4.1) Inhibition of proliferative signaling

I.8.4.1.1) Targeting ErbB family

Epidermal growth factor receptors are members of the transmembrane tyrosine kinase receptors family. Once activated the signal transduction cascades promote cellular proliferation and survival through two main pathways: Ras/raf/MAPK and PI3K/Akt/mTOR. Briefly ErbB family receptors need to dimerize through homo or hetero dimerization for tyrosine kinase activation. The most important ErbB family member in breast cancer is ErbB2. Interestingly in contrast to other ErbBs, ErbB2 can adopt a constitutively activated state and is the preferred dimerization partner of ErbB receptors. Moreover this receptor is overexpressed in 20% to 30% of breast cancer suggesting that ErbB2 is a major target for breast cancer. ErbB2 importance in breast cancer and description is summarized later in this thesis (Hudis, 2007; Fang et al., 2011a; Wicki and Rochlitz, 2012).

Trastuzumab (Herceptin; Genetech/Roche, South San Francisco, CA) is the first anti-ErbB2 targeted agent developed in breast cancer. ErbB2 extracellular domain (ECD) is composed of four subdomains and the Trastuzumab Fab binds to the domain IV. This juxtamembrane binding site of Trastuzumab on ErbB2 receptor results in: a) reducing shedding of the extracellular domain; b) into a physical inhibition of receptor dimerization; c) the activation of an immune response by recruiting immune effector cells leading to tumor cell death; and d) receptor down-regulation through endocytosis (Hudis, 2007). Nevertheless, this multiple levels of mechanism of action of trastuzumab doesn't overcome numerous mechanisms of resistance and about 30% to 50% of ErbB2 over-expressing breast cancers are primary resistant to Trastuzumab. According to Fang et al., many mechanisms can alter the complex ErbB2-trastuzumab: a) the antibody binding site can be masked or block by cleavage of ErbB2 ECD or overexpression of MUC4 (Mucin-4 a membrane ErbB2 ligand); b) ErbB2 downstream signaling can be altered through down regulation of phosphatase and tensin homolog (PTEN) tumor suppressor gene and PI3K mutant activation enhancing ErbB2 signaling network; c) inhibition of cell cycle growth arrest by the loss of cyclin-dependent kinase inhibitor p27; d) compensation mechanism by signaling though other ErbB receptors or non-ErbB receptors such as insulin growth factor receptor (IGFR). Additionally the most common side effect of Trastuzumab is cardiotoxicity characterized by a decrease of left ventricular ejection fraction (LVEF) (Wicki and Rochlitz, 2012).

To improve trastuzumab efficacy, the antibody was conjugated to the fungal toxin DM1: Trastuzumab-maytansine = T-DM1 (Genentech/Roche, South San Francisco, CA). DM1 is a highly potent anti-microtubule agent inhibiting their assembly. Here trastuzumab is used as a carrier delivering DM1 to the tumor cells harboring ErbB2 receptor. Targeting microtubules, this conjugated antibody can therefore overcome some resistant mechanism of the antibody alone (Fang et al., 2011a; Wicki and Rochlitz, 2012). Here again treatment can lead to diverse toxic effects including thrombocytopenia, anemia and neuropathy (Chu and Lu, 2008b).

Pertuzumab (Omnitarg^{TM}; Genetech/Roche, South San Francisco, CA) is also a humanized antibody targeting another ECD of ErBB2, the dimerization arm within the subdomain II. The antibody binding site, which is quite different from Trastuzumab, sterically blocks homo and hetero-dimerization of ErbB2 thereby inhibiting downstream signaling transduction (Fang et al., 2011b; Wicki and Rochlitz, 2012). Diarrhea, pain, nausea, vomiting are some of the side effects observed with Pertuzumab (Chu and Lu, 2008b).

Ertumaxomab (Rexomun^{TM}; Fresenius Biotech, Hamburg, DE) is a tri-functional antibody targeting ErbB2, CD3 on the T cells, and Fc receptors. In presence of Ertumaxomab, tri-cell complexes consisting of tumor cells, T cells, and accessory cells (macrophage, natural killers) form to cause the aggregation of these cells leading to tumor cell death through phagocytosis (Fang et al., 2011).

The ADAMs (a disintegrin and metalloproteinase) are members of the superfamily of matrix metalloproteinase, they are implicated in different biological functions such as adhesion, migration, proteolysis and cancer. These molecules harbor a sheddase activity allowing the cleavage of extracellular domains of transmembrane receptors at their juxtamembrane domain thereby releasing bound-ligands (Duffy et al., 2009). In breast cancer ADAM-10 and ADAM-17 have exhibited sheddase activity on the ErbB family. Indeed by cleaving the extracellular domain of ErbB2 ADAMs form the truncated ErbB2 protein p95HER2 (amplifying the intracellular kinase activity) as well as releasing multiple ErbB ligands. ADAM-10 and ADAM-17 are inhibited with INCB7839 (Incyte corporation, Wilmington, DE) which inhibits sheddase activity and prevent the formation of the oncoprotein p95HER2. This compound is in phase II and has demonstrated good tolerance and promising activity in HER2 positive metastatic breast cancer (Fang et al., 2011).

HSP90 (heat shock protein 90) chaperones stabilize, activate, protect from misfolding and degradation of multiple proteins, including ErbB2, leading in breast cancer to cell survival. Inhibiting HSP90 therefore decreases ErbB2 expression at the cell surface. Tanespimycin

Network targets	Agent	Company	Clinical trials	NCT
PIK3CD	CAL-101	Calistoga Pharmaceuticals, Inc.	Phase II	NCT00710528
PIK3CA	BYL719	Novartis	Phase I	NCT01219699
PI3K selective inhibitors	PX-866	Oncothyreon Inc	Phase I	NCT00726583
	GDC-0941	Genentech Inc	Phase II	NCT00960960
	XL-147	Exelixis	Phase I/II	NCT01042925
	BKM-120	Novartis	Phase I	NCT01068483
Multitarget inhibitor Pan-PI3K, mTORC1/2, DNA-PK, HIF-1α	SF1126	Semafore Pharmaceuticals	Phase I	NCT00907205
Dual PI3K/mTOR	XL-765	Exelixis	Phase II	NCT01082068
	BEZ-235	Novartis	Phase I	NCT00620594
	GSK1059615	GlaxoSmithKline	Phase I	NCT00695448
AKT inhibitors	Perifosine	Keryx/AOI Pharmaceuticals	Phase II	NCT00847366
	GSK690693	GlaxoSmithKline	Phase I	NCT00493818
	MK-2206	Merck	Phase II	NCT00963547
Rapamycin analogs	Sirolimus	Wyeth/Pfizer	Phase II	NCT00411788
	Temsirolimus	Wyeth/Pfizer	Phase II	NCT01111825
	Everolimus	Novartis	Phase II/III	NCT01061788
	Ridaforolimus	ARIAD/Merck	Phase II	NCT01234857
mTOR kinase inhibitors	AZD-8055	AstraZeneca	Phase I	NCT00973076
	OSI-027	OSI Pharmaceuticals	Phase I	NCT00698243
	INK-128	Intellikine	Phase I	NCT01058707

Abbreviations: DNA-PK, DNA-dependent protein kinase catalytic subunit; HIF-1α, hypoxia-inducible factor 1, α subunit; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PIK3A, phosphoinositide-3-kinase; α polypeptide; PIK3CD, phosphoinositide-3-kinase, catalytic, δ polypeptide.

Table 7: Agents targeting the PI3K/Akt/mTOR pathway included in clinical trials. PI3Ks are grouped into classes I, II or III, containing each of them different isoforms. For example, the class IA PI3K includes PIK3CA, PIK3CB and PIK3CD (Hernandez-Aya and Gonzalez-Angulo, 2011).

(Telatinib™/17-AAG; Kosan Biosciences, Hayward, CA) binds to the ATP pocket of HSP90 and block its chaperone function. Results of phase II study revealed promising activity in HER2 metastatic breast cancer (Fang et al., 2011; Wicki and Rochlits, 2012).

Besides targeting the extracellular domain of ErbB2 numerous compounds target the intracellular domain or downstream signaling of ErbB2 receptor. Several tyrosine kinases inhibitors (TKI) have been developed including the well-known Lapatinib (Tykerb™, GlaxoSmithKline, Research triangle Park, NC) and Neratinib (HKI-272, Wyeth Corp, Madison, NJ). Lapatinib is directed against both HER2 and HER1 and binds reversibly to the ATP pocket inhibiting receptor auto-phosphorylation. Similar to Lapatinib, Neratinib also inhibits receptor auto-phosphorylation (irreversely) but of a wider range of ErbB receptors; HER1, HER2 and HER4 (Fang et al., 2011; Wicki and Rochlits, 2012). Here again diarrhea, rash nausea and vomiting can be observed under treatment (Chu and Lu, 2008b).

I.8.4.1.2) Targeting ErbB signaling

The PI3K/Akt/mTOR pathway comprises many potential targets for drug development in breast cancer, table 7 summaries current therapies. More than 70% of breast tumors have molecular alteration in at least one component of the pathway such as PI3KCA-activating mutation (Hernandez-Aya and Gonzalez-Angulo, 2011).

I.8.4.2) Interfering with DNA repair pathways: Synthetic lethality by inhibiting poly-ADP-ribose polymerase (PARP) in breast cancer susceptibility gene 1 and 2 (BRCA1, BRCA2) deficient cells

The major forms of DNA damage include SSB (single-strand breaks) and DSB (double strand breaks). Single strand breaks repair depends on poly-ADP-ribose polymerase (PARP) and the most important mechanism for repair of DNA double strand breaks is homologous recombination (HR). BRCA1 and BRCA2 are tumor suppressor genes and play a key role in the process of HR repair. Importantly, hereditary breast cancer accounting for 5 to 10% of all breast cancers, is chiefly attributed to BRCA1 and BRCA2 mutation making such cells important targets in hereditary breast cancer. In BRCA1/2 mutated cells, were HR is impaired, the inhibition of

Therapy type	*Medication*	*Typical course of treatment*
Chemotherapy[24]	**Anthracyclines**	
	Doxorubicin (Adriamycin)	IV every 14 to 21 days for four to six cycles; used in combination with a taxane (docetaxel [Taxotere] or paclitaxel [Taxol]), cyclophosphamide, and/or fluorouracil
	Epirubicin (Ellence)	IV day 1 or days 1 and 8, every 21 to 28 days for three to eight cycles; used in combination with cyclophosphamide or fluorouracil
	Taxanes	
	Docetaxel	IV every 21 days for three to four cycles; used in combination with doxorubicin, epirubicin, cyclophosphamide, and/or fluorouracil
	Paclitaxel	IV every seven to 21 days for four to 12 cycles; used in combination with doxorubicin and cyclophosphamide
Endocrine	**Aromatase inhibitors**	
	Anastrozole (Arimidex)	Oral tablet daily for five years; used alone or in sequence with tamoxifen[36,37]
	Exemestane (Aromasin)	Oral tablet daily for at least two to five years; used alone or in sequence with tamoxifen[38,39]
	Letrozole (Femara)	Oral tablet daily for two to five years; used alone or in sequence with tamoxifen[40,41]
	Gonadotropin-releasing hormone agonist	
	Goserelin (Zoladex)	Subcutaneously every one to three months for two years[42,43]
	Selective estrogen receptor modulators	
	Tamoxifen	Oral tablet daily for two to five years; used alone or in sequence with an aromatase inhibitor[36]
Tissue-targeted	**Monoclonal antibody**	
	Trastuzumab (Herceptin)	IV with first dose of chemotherapy regimen and then every one or three weeks to complete one year[44-46]

IV = intravenously.

Table 8: Common medications and typical course of treatment used in breast cancer (Maughan et al., 2010a)

PARP will induce the conversion of SSBs into DSBs. The accumulation of DSBs will form a complex lethal chromosome alteration inducing cell death. Olaparib (AZD2281; AstraZeneca, London, UK) and Iniparib (BSI-201, BiPar Sciences, Inc) are both PARP inhibitors in human phase I study and exhibit promising antitumor activity (Fang et al., 2011; Wicki and Rochlits, 2012).

I.8.4.3) Anti-angiogenic therapies

Angiogenesis is the formation of new vessels from pre-existing ones and is an essential step for breast cancer progression and dissemination (Filho et al., 2010). Vascular epithelial growth factor (VEGF) is a crucial mediator in angiogenesis and overexpression has been linked to the amplification of the oncogene HER2. The first anti-angiogenic therapy approved by the Food and Drug Administration (FDA) is Bevacizumab (Avastin^{TM}; Genetech/Roche, South San Francisco, CA) which binds the vascular endothelial growth factor A (VEGF-A) thereby inhibiting VEGFR activation and neovascularization enhancing signal. This anti-angiogenic therapy exhibited substantial benefit in the treatment of metastatic breast cancer. Typical course of treatment of Bevacizumab is presented in table 8. Unfortunately, hypertension, proteinuria, bleeding are major side effects observed with Bevacizumab therapy (Chu and Lu, 2008b). Down-stream signaling through VEGFR tyrosine kinase activity can be inhibited by Sorafenib (Nexavar^{TM}; Bayer Health Care AG, DE) and Sunitinib (Sutent^{TM}; Pfizer, New York, NY) two tyrosine kinase inhibitors (TKIs) (Fang et al., 2011; Wicki and Rochlits, 2012).

When analyzing drug development the actual trend is to develop novel candidates with larger spectrum of action, inhibiting signaling platforms rather than inhibiting a single target; indeed Pertuzumab has been develop to inhibit both homo and heterodimerization of ErbB2, moreover recent TKIs have also wider spectrum of action has evaluated with Neratinib inhibiting HER1, HER2 and HER4 compared to Lapatinib. As mentioned above a sobering fact is that numerous therapies lead in turn to resistance mechanism and toxicity which highlights the vital need for novel targeted drugs to fight breast cancer and metastases. Currently, common therapies target the extracellular domain and the intracellular domain (figure 8) but inhibiting transmembrane domain (TMD) of bitopic receptors still largely remains terra incognita. In breast cancer drug

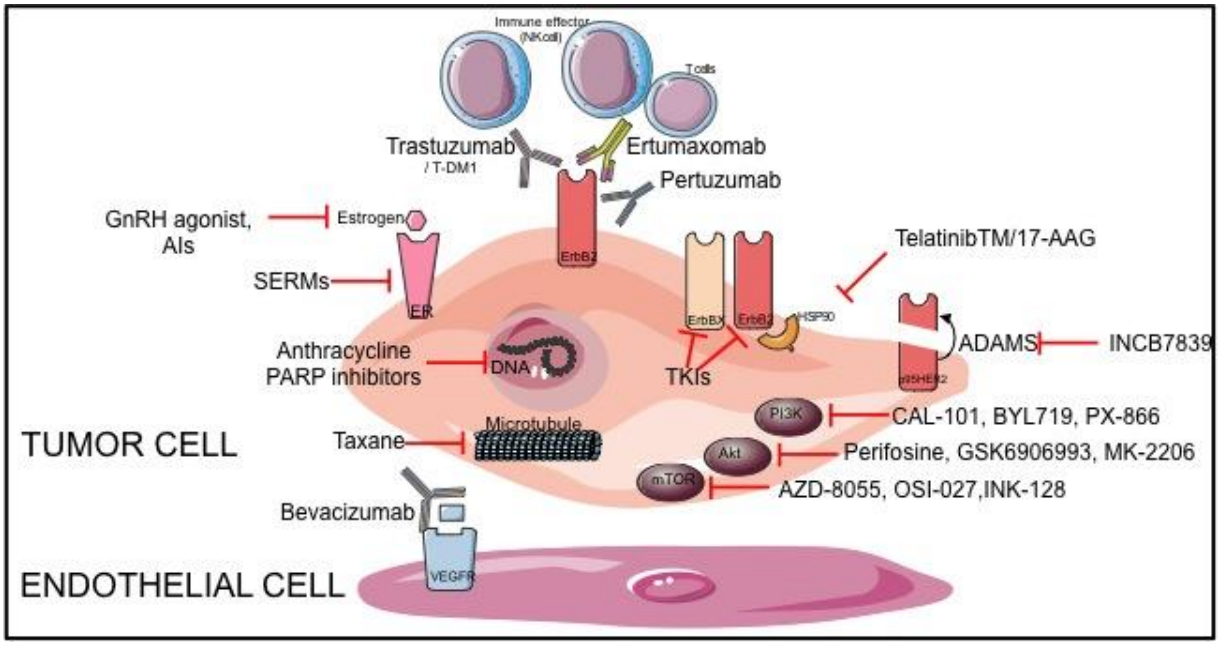


Figure 8: Schematic representation of targets and treatments used in the treatment of breast cancer

development one should focus on new strategies targeting signaling platforms applied to validated therapeutic target such as ErbB2 and widen research to other potential therapeutic targets.

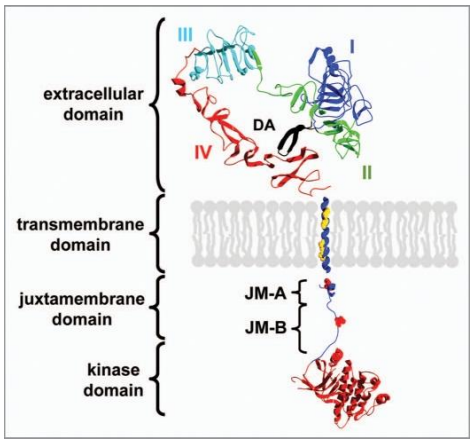


Figure 9: Domain structure of ErbB receptors. The extracellular domain is composed of four subdomains (I–IV) and the dimerization arm (DA). The intracellular domain is composed of the justamembrane domain –A and –B (JM-A and JM-B), and the tyrosine kinase domain (Cymer and Schneider, 2010

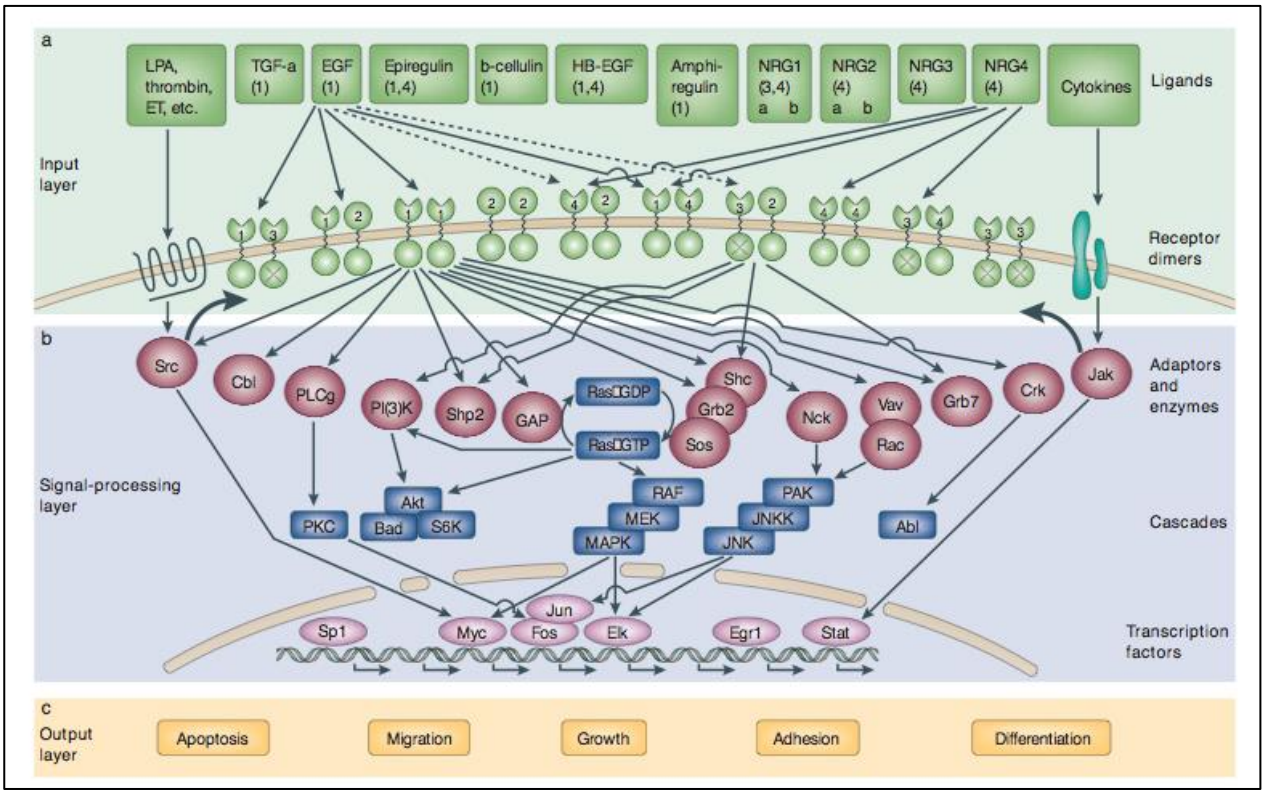


Figure 10: ErBb signaling network (Yarden et al., 2001)

II) Therapeutic targets in breast cancer: validated and future targets

II.1) Human epidermal growth factor receptor 2 (HER2)

II.1.1) Signaling network platform

Human epidermal growth factor receptor 2 (HER2), is a bitopic transmembrane receptor member of the human epidermal growth factor family (HER/ErbB) which includes four receptors; HER1 (EGFR), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). Each receptor consists of an extracellular domain (ECD) corresponding to the ligand binding site (excepted in the case of ErbB2), a transmembrane domain (TMD) and, apart HER3, a functional intracellular tyrosine kinase domain. HER2, a 185kDa glycoprotein, is the only receptor with no clear identified ligand (Tai et al., 2010) (figure 9). Carraway and collaborators propose MUC4, a membrane mucin, as a novel intramembrane ligand for HER2 (Carraway et al., 2003). Due to an intrinsically extended interaction loop, HER2 is the preferred dimerization partner of all family members and harbors the strongest catalytic kinase activity leading to very potent signaling functions among all dimers. There are 10 possible combinations of ErbBs and at least 13 recognized ligands (figure 10): epidermal growth factor (EGF), heparin-binding (HB)-EGF, transforming growth factor alpha (TGFα), amphiregulin (AREG), epiregulin (EREG), epigen (EPG), betacellulin (BTC) and neuregulins (NRG 1-6). EGF ligands bind preferentially on one or the other ErbB receptor. Indeed they can be divided into three groups: the first includes EGF, TGFα and AREG, which binds specifically to ErbB1 and the second includes BTC, HB-EGF and EREG, which shows dual specificity binding both ErbB1 and ErbB4. The third group is constituted of the NRGs and forms two subgroups based on their capacity to bind ErbB3 and ErbB4 (NRG-1 and NRG-2) or only ErbB4 (NRG-3 and NRG-4). Interestingly EGF ligands exist as membrane-anchored precursors and are cleaved by metalloproteases (mainly ADAMS), indeed their ectodomains are processed by proteolysis leading to shedding of the soluble factor (Tai et al., 2010; Yarden, 2001; Eccles, 2011a).

Structurally, HER2 ECD can be divided into four subdomains. The theoretical binding site for receptor ligands is composed by the subdomain I and III (figure 9). Subdomains involved in the homodimerization and heterodimerization are domains II and IV. As presented above

subdomain IV is the binding site of Trastuzumab whereas subdomain II exhibiting a dimerization arm (the important contributor for dimerization) is the target of Pertuzumab (Tai et al., 2010).

Ligand binding to ErbBs induces formation of various homo and hetero dimers. Consequently the intrinsic kinase domain is activated resulting in the phosphorylation of tyrosine kinase residues in the cytoplasmic domain of the receptor. These phosphorylated residues serve as docking sites for a variety of signaling molecules leading to the activation of intracellular pathways. Transduction of HER2 signal is achieved through two major pathways: the Ras/Raf/MAPK/ERK and the PI3K/AKT/mTOR pathways, but also via the phospholipase C and signal transducer and activator of transcription (STAT) pathway resulting in a plethora of biological functions. Profiling of phosphotyrosin interaction sites has shown that the 10 possible combinations of ErbBs receptors have specific pattern of binding partners (figure 10). Therefore, HER2 signaling leads in turn to cell growth, survival and differentiation in a complex manner (Holbro and Hynes, 2004; Tai et al., 2010; Eccles, 2011b).

The structural basis for receptor dimerization has come to light thanks to crystallographic data. Importantly they are two conformations of the extracellular domain, a closed inhibited or an open active conformation. Ligand binding changes the receptor conformation and induces the active conformation and transphosphorylation. Here the association between subdomain I and III leads to the protrusion of the dimerization arm making it fully accessible for dimerization (Tai et al., 2010). In contrast with other ErbB receptors, most HER2 receptors exist naturally in an activated conformation, which is consistent with the lack of known ligands. Having this in mind, with the lack of functional tyrosine kinase activity of HER3, HER2 and HER3 harbor therefore both incomplete signaling. This heterodimer forms the most active signaling and common heterodimer of the HER family (Moasser, 2007a).

II.1.2) Roles during embryogenesis and adulthood

As mentioned above HER2 lacks ligand binding and needs to dimerize to trigger intracellular signaling with other HER receptors: HER1, HER3 and HER4. Additionally, as HER2 is the favorite partner for dimerization, all together these observations place HER2 as pivotal receptor in the

ErbB signaling platform. Therefore studying HER2 function during embryogenesis and adulthood implies to take into account HER2 dimerization partners HER1, HER3 and HER4 as well.

The importance of ErbBs function and roles during embryogenesis has been assessed through the generation of mice lacking one or the other ErbB receptors. Inactivation of EGFR leads to a variety of phenotypes, indeed mice surviving for up to 8 days after birth suffer from impaired epithelial development in several organs, including skin, lung, gastrointestinal tract and in some regions of the brain (Miettinen et al., 1995; Holbro and Hynes, 2004). In contrast to ErbB1 -/- mice, ErbB3 -/- embryos only survive until E13.5. Here again numerous defects are observed which include cardiac cushion abnormalities leading to blood reflux through defective valves, midbrain and hindbrain regions are affected and cranial ganglia defects are detected (Erickson et al., 1997). Mice lacking ErbB4 die during mid-embryogenesis from the aborted development of myocardial trabeculae in the heart ventricle. Cardiac trabeculae are distinct muscle tracts connecting between different local areas of the inner heart wall, particularly at the apex and septal junctions. ErbB4 -/- mice also display alterations in the innervation of the hindbrain in the central nervous system (Gassmann et al., 1995). Remarkably, ErbB2-/- embryos die on E10.5 due to a lack of cardiac ventricular myocyte differentiation and trabeculation defect. They also exhibit cranial ganglia defects as observed in the ErbB3 lacking mice (Erickson et al., 1997). According to Morris, ErbB2 play as well an essential role in peripheral nervous system development (Morris et al., 1999).

The fact that all null mutations in the ErbB signaling system are lethal, the study of the specific roles of these receptors in later stages of development is difficult. To circumvent this issue, conditional knockout mice in a define organ were engineered. Interestingly, a mutant mouse line carrying a cardiac-restricted deletion of ErbB2 exhibited a dilated cardiomyopathy resulting in a reduced contractile function of the heart (Negro et al., 2004). Conditional knockout in skeletal muscles leads to a defect in muscle regeneration (Andrechek et al., 2002; Holbro and Hynes, 2004). Another study revealed the importance of ErbB2 in the formation of effective neuromuscular synapses. Additionally, mice deficient of ErbB2 in Schwann cell exhibit movement abnormalities (Holbro and Hynes, 2004).

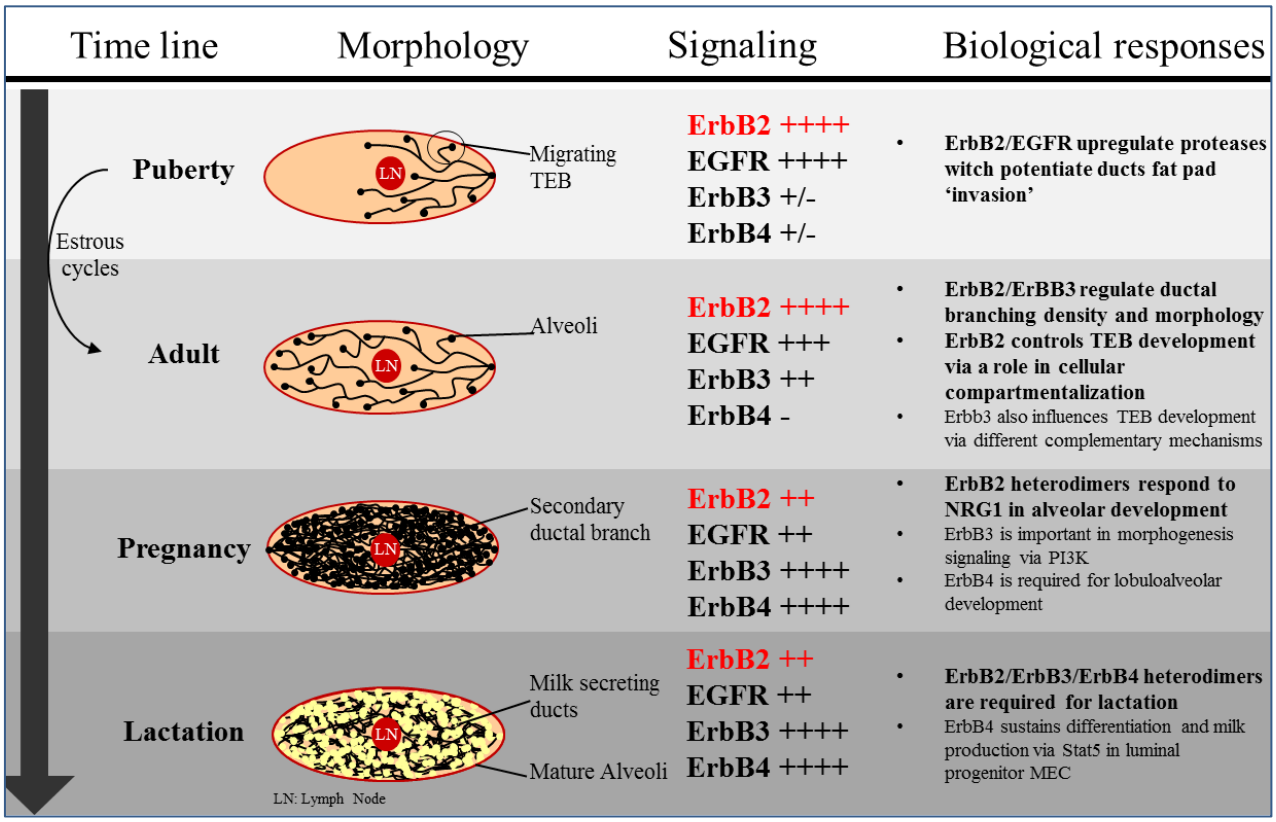


Figure 11: ErbB family in normal breast biology adapted from Eccles (2011)

In brief, EGFR is essential for the maintaining of skin and squamous epithelia and HER2, HER3 and HER4 are important in the cardiovascular and nervous system. These numerous studies underline the fact that HER proteins are essential in mammalian development and involved in the function of diverse organs including the breast, brain, skin, lung and gastrointestinal tract.

Importantly, ErbB2 protein levels are absent or low in most normal adults tissues with the exception of the heart explaining the observed cardiac toxicity of Trastuzumab and Pertuzumab (intensified with antharcyline chemotherapy) (Eccles, 2011b).

II.1.3) Functions in the mammary gland

The ErbB network has been deeply studied in the mammary gland that undergoes most of its development after birth as mentioned above. Here again rodent models allowed determining ErbB roles in normal breast development. This organ is particularly interesting because of its susceptibility to HER2 induced cancer. Interestingly, mutant mice do not exhibit severe embryonic mammary gland defects suggesting a degree of redundancy between receptors and ligands (Hardy et al., 2010). Indeed structural defects in TEBs have been observed in conditional ErbB2-/- mammary epithelium mice. ErbB receptors are all expressed at some time during mammary gland development and maturation, and play complementary role as presented in the figure 11. Signaling between stromal mammary cells and epithelial mammary cells is very important. EGF receptors ligands are upregulated mainly during puberty, pregnancy and lactation; this implies a major role of ErbB network during those phases. ErbB2 is expressed during all periods of development and maturation, which is not observed for ErbB4. During puberty, ductal outgrowth and TEB development are directly linked to ErbB2 presence in the epithelial mammary cells, involving ErbB3 as the main partner for signaling. In the maturation phase of the gland ErbB2 and ErbB3 regulate ductal branching density and morphology. During pregnancy and lactation ErbB2 is required for alveolar differentiation and milk protein production (Eccles, 2011b).

Since these ligands and receptors are involved in proliferation, invasion, differentiation and growth, it is not surprising that many of these proteins are highly expressed in breast cancer with their importance being ranked such as ErbB2 > ErbB1 > ErbB3 and ErbB4 (Eccles, 2011b).

II.1.4) Protein Regulation

Regulation of HER2 protein is of particular importance as HER2 amplification is reported in 20 to 30% of human breast cancer. The causative factors responsible for increasing HER2 expression levels on mammary cells are not fully understood and only very few studies have investigated this issue. Nevertheless, matrix metalloprotease-9 (MMP-9), adipocyte protein 2 (AP2), polyoma enhancer activator protein 3 (PEA3), retinoblastoma (RB) tumor suppressor, and estrogen receptor have been suggested as potent HER2 regulators. MMP-9 increases HER2 expression in human mammary epithelial cells, as the induction in HER2 protein expression was suppressed using a MMP-9 inhibitor (Fatunmbi et al., 2012). AP2, a carrier protein for fatty acids, has been shown to be a strong activator of HER2 gene, as HER2 protein expression is correlated with the activity of AP2 (Alroy and Yarden, 1999). On the other hand HER2 is also subjected to negative regulation of various factors such as PEA3 and the retinoblastoma tumor suppressor (RB) (Hung and Wang, 1999). PEA3 is a member of the ETS (E-twenty six) oncogene transcription factor family; this protein is overexpressed in oncogene-induced mouse mammary tumors and metastatic human breast cancer cells (Chotteau-Lelievre et al., 2003). Moreover, estrogen-dependent stimulation of estrogen receptor negatively regulates the expression of HER2 at protein and mRNA level (Russell and Hung, 1992). Further studies need to be investigated to achieve a complete picture of HER2 protein regulation.

II.1.5) Variants, homologue and nomenclature

In naturally occurring human breast cancer, three HER2 spliced variants in breast cancer have been reported to date. HER2 mRNA contains 27 exons and 26 introns (Wan et al., 2009). Δ16HER2 lacks a single 48bp coding region resulting in the in-frame deletion of exon 16, encoding a region in the extracellular domain of HER2. This deletion leads to a conformational change of HER2 protein promoting homodimerization. Δ16HER2 appears to be more aggressive than the wild type HER2 and is implicated in anti-HER2 therapies resistance. P100 and herstatin are two other naturally spliced variant of HER2. Both are proteins constituting the extracellular domain of HER2 protein but herstatin harbors a C terminus added of 79 amino acids. In contrast to Δ16HER2, p100 is inversely correlated with aggressiveness. Herstatin is able to interfere with dimerization as an inhibitor of full length HER2 protein. Such as p100, this variant harbors a

Name	Description	Structure	Species
ErbB2	Include human and rodent epidermal growth factor receptor protein or gene	–	In human and rodent cells
HER2	Human cellular epidermal growth factor receptor protein	–	Only in human cells
Neu, c_neu, wtneu	Rodent cellular homologue of epidermal growth factor receptor protein	–	Only in rat and mouse cells
Δ16HER2	Human epidermal growth factor receptor spliced variant	Extracellular HER2	In human breast cancer
p100	Human epidermal growth factor receptor spliced variant	Extracellular HER2	In human breast cancer
Herstatin	Human epidermal growth factor receptor spliced variant	Extracellular HER2	In human breast cancer

Table 9: Nomenclature of HER2 human and rodent homologue variants

“protective” profile and is therefore inversely correlated with tumor aggressiveness. Other truncated proteins are detectible in human breast cancers but they don’t occur naturally but rather though proteolytic cleavages, as observed with p95HER2 produced by ADAMs discussed earlier (Jackson et al., 2013).

Importantly HER2 has a rodent homologue called Neu. The Neu oncogene was first identified in a chemically-induced neuroblastoma (Shih et al., 1981). Extended details on Neu homologue will be presented further in this thesis in the material and methods section. Common nomenclature of HER2 human and rodent homologues variants are presented in the table 9.

II.1.6) Transcriptional targets

Besides the protein kinase activity, numerous studies proposed a nuclear function as a transcription factor for HER2. Transcriptional targets can themselves enhance HER2 tumorigenic signaling resulting in a vicious circle. Nuclear HER2 was found to associate with multiple genomic targets, the induction of CXCR4 (CXC chemokine receptor 4), COX-2 (cyclooxygenase 2) and VEGF are the most potent suggested targets. CXCR4, a chemokine receptor, is positively correlated with HER2 in breast cancer and metastasis progression. Furthermore, HER2 inhibits CXCR4 ubiquitination and therefore degradation. Additionally, HER2 phosphorylation is increased when CXCR4 is stimulated by its ligand (SDF-1 stromal derived factor-1) (Moasser, 2007b; Tai et al., 2010). As CXCR4, COX-2 expression in breast tumor tissues correlates closely with nuclear expression of HER2. COX-2 is an enzyme which synthetizes prostaglandins from the conversion of arachidonic acid (AA) during inflammation process. Here HER2 forms a complex with the COX-2 promoter and is able to stimulate its transcription (Wang et al., 2004). Suppressing COX-2 in HER2 overexpressing cells results in inhibition of the invasion activity, suggesting here as well an implication of COX-2 in metastasis progression (Wang et al., 2004). As mentioned above, vascular epithelial growth factor (VEGF) is a crucial mediator in angiogenesis and its overexpression has been linked to the amplification of the oncogene HER2. Indeed in a clinical study, 87.7% of HER2 positive breast cancers were associated with VEGF overexpression (Konecny et al., 2004). Of course, the role of many other genes need to be studied and identified.

II.1.7) Validated target in breast cancer and MBC

HER2 overexpression is observed in 20 to 30% of breast cancer and is often a consequence of gene amplification, which appears to induce spontaneous dimerization without need of a ligand. Indeed 25 to 50 copies of the ErbB2 gene can be observed in breast cancer cells leading to 2 million of receptors per cell (Eccles, 2011b). Thereafter having in mind that HER2 expression is low or absent in adults, accumulating evidences point out HER2 as a logical selective target for breast cancer therapy. Moreover, HER2 overexpression is associated with poor prognosis, shorter survival rates and relapse time, greater lymph node invasion and more aggressive tumor phenotypes. Evidences suggest that ErbB2 amplification in rather an early event in tumorigenesis and appears in half patients presenting DCIS (Tai et al, 2010; Eccles, 2011b). In humans ErbB2 overexpression is also reported in other cancers, such as gastric, ovarian and prostate cancer (Holbro and Hynes, 2004).

The occurrence of metastasis is a critical step in breast cancer progression and linked to disease-associated death. Indeed as exposed earlier the probability of death from localized breast cancer (in situ breast cancer) ranges from 0.03 to 0.1 and this probability is considerably worsen for patient with distant disease, 0.70 to 0.85 (Schairer et al., 2004). The common sites of breast cancer metastasis include bone, brain, lung and liver. According to Eccles in 2011, HER2 is involved in all the different steps of metastasis from the primary tumor site to distant secondary sites. HER2 regulates factors that promote angiogenesis, invasion, motility, directional migration, extravasation and protection from anoikis (Eccles, 2011b). In numerous studies, positive breast cancers for HER2 receptor have been associated with increase risk for brain metastases but a conflicting study suggests no association (Grewal and Kesari, 2008). Nevertheless, Palmieri observed that HER2 overexpression increases the metastatic outgrowth of breast cancer cells in the brain and suggests here an implication of EGFR as a major co-receptor of HER2 in brain metastasis development (Palmieri et al., 2007). According to Eccles, HER2/HER3 heterodimer is also of importance in brain metastasis development. Interestingly, this review points out the fact that this heterodimer responds to NGRs, which are local neural growth factors. Besides the brain, a common signaling mechanism leading to lung, bone and liver metastasis correlated with HER2 protein has been studied. CXCR4, a chemokine receptor,

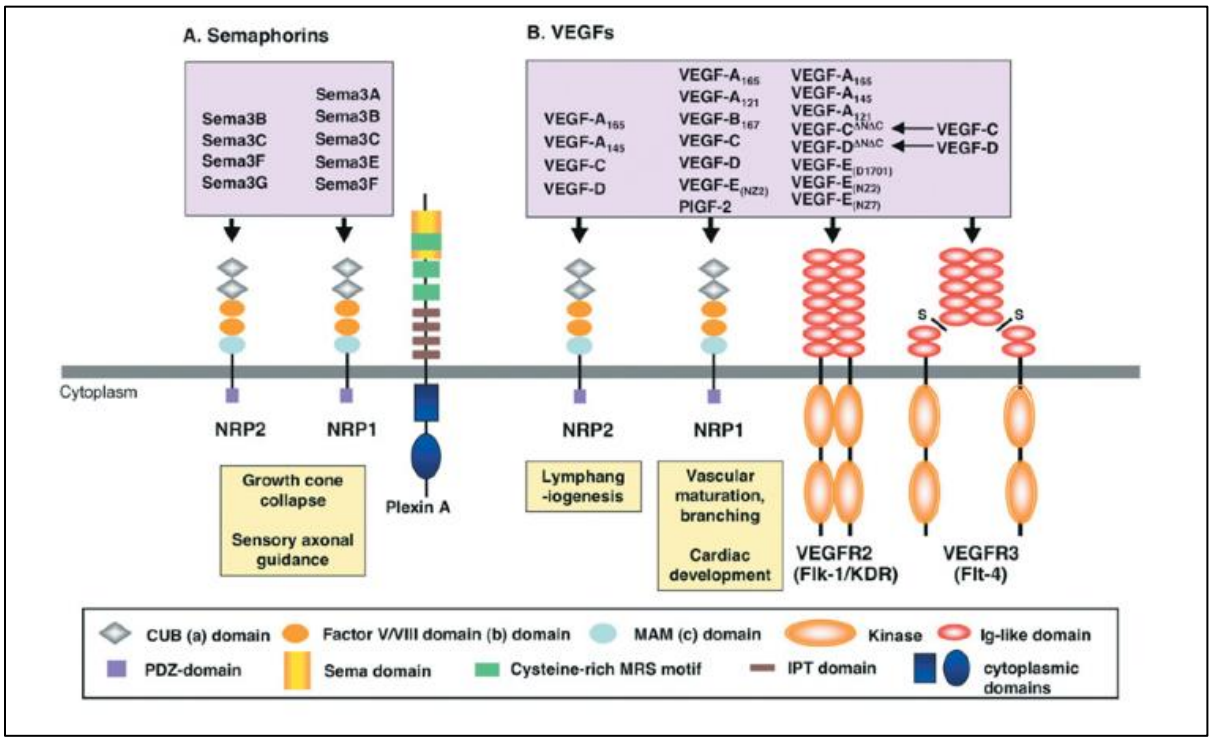


Figure 12: Neuropilins, their ligands and co-receptors (Pellet-many, 2008)

and its ligand the stromal cell derived factor -1 alpha (SDF-1) are implicated in metastatic processes in these sites. Notably, there is a correlation between HER2 and CXCR4 in breast tumor tissues proposing a possible signaling pathway through which HER2 positive breast cancer cells could metastasize to these organs (Freudenberg et al., 2009).

Overall findings are consistent with the fact that HER2 is a validated therapeutic target in breast cancer and its related metastasis.

II.2) Neuropilins: neuropilin-1 (NRP1) and neuropilin-2 (NRP2)

II.2.1) Signaling network platform

Such as HER2, neuropilins (neuropilin-1 (NRP1) and neuropilin-2 (NRP2)) are bitopic transmembrane glycoprotein receptors composed of an ECD, a TMD and an intracellular domain, but neuropilins are non-tyrosine kinase receptors. They are 120 to 130 KDa glycoproteins with multiple co-receptors. Indeed neuropilins are thought to transduce functional responses only when co-expressed with other receptors, these include vascular endothelial growth factor receptors (VEGFR) and plexins. The most known ligands of neuropilins are class 3 semaphorins and VEGF, which are of particular interest in cancer, but also at various degrees heparin binding proteins, fibroblast factor-2 and placental growth factor. Due to the binding of diverse ligands, neuropilins exhibits multiple biological functions such as axonal guidance in the developing nervous system and in vascular development (Ellis, 2006). Semaphorins (SEMA) are divided into 7 classes containing more than 20 proteins and class 3 to 7 belong to vertebrate. They are membrane bound and cleaved by proteolytic degradation. Class 3 semaphorins require NRP1 and 2 as obligate receptors and have different affinities for one or the other receptor (see figure 12); neuropilin-1 is a receptor for SEMA3A, SEMA3B, SEMA3C, SEMA3E and SEMA3F and neuropilin-2 rather binds SEMA3B, 3C, 3F, 3G (He and Tessier-Lavigne, 1997; Bagri and Tessier-Lavigne, 2002a; Chen et al., 1997). In tumorigenesis, semaphorins exhibit pro and anti-tumoral effects (Nasarre et al., 2005; Gu and Giraudo, 2013; Neufeld et al., 2012). VEGF plays an important role in vasculogenesis and angiogenesis. In mammals, VEGF family comprises 4 members VEGF -A, -B, -C and -D in which three members bind neuropilins, VEGF-A, VEGF-B and VEGF-C. VEGF-A occurs in at least six different isoforms including VEGF-

165, the most active and abundant isoform. Here again VEGFs binds preferentially one or the other neuropilins as presented on the figure 12 (Pellet-Many et al., 2008).

The basic structure of neuropilins comprises five domains: three extracellular domains, 1) the CUB (complement C1r/C1s, Uegf, Bmp1) homology domains (a1 and a2), binding sites of semaphorins, 2) two coagulation factor V/VIII homology domains (b1 and b2) binding sites of VEGF, and 3) a MAM (meprin, A-5 protein, and receptor protein-tyrosine phosphatase mu) or c domain critical for homodimerization and heterodimerization with co-receptors, 4) a transmembrane domain, and 5) a short cytoplasmic domain. As mentioned above the cytoplasmic domain does not harbor a tyrosine kinase motif but a PDZ motif (PSD-95/Dlg/ZO-1) potentially serving as a docking site for intracellular signaling through synectin for example (Pellet-many et al., 2008).

Signaling transduction for neuropilins implies an association with a co-receptor upon ligand binding. Plexins are transmembrane proteins and nine members have been identified further subdivided into four families: plexin-A (1-4), plexin-B (1-3), plexin-C1 and plexin-D1 (Tamagnone et al., 1999). Structurally, plexin are composed of 1) an ECD comprising a sema domain followed by two or three MRS domains (Met-related sequences rich in cysteins) and three or four IPTs domains (immunoglobulin-like domains chaired by plexins and transcription factors), 2) a TMD and 3) an intracellular domain containing two conserved regions responsible for downstream signaling, occurring mainly through the activation of Rho-like GTPases. Neuropilin/Plexin complex can form, through ligand binding, an extracellular bridge, leading in turn to growth cone collapse and the regulation of sensory axonal guidance. Such as plexins, VEGFRs are co-receptors for neuropilins and most of the past work focused on neuropilin-1 associations with VEGFR2, nevertheless the existence of NRP2/VEGFR-1, -2 and -3 have been described such as NRP1/VEGFR-1 and -3. VEGFRs transmembrane proteins are also composed of three regions, an extracellular domain composed of immunoglobulin like domains, a TM domain and an intracellular domain harboring tyrosine kinase activity. Downstream signaling through VEGFRs includes the PLC-y/PKC/ERK1/2 and the PI3K/Akt pathway leading to cell migration, angiogenesis, lymphangiogenesis, cardiac development and vascular remodeling (Ellis, 2006; Pellet-Many et al., 2008; Grandclement et al., 2011a). Besides the fact that ligand binding forms

an extracellular bridge between both molecules, neuropilin cytoplasmic PDZ domain could be here again important for the complex formation (Prahst et al., 2008).

II.2.2) Roles during embryogenesis and adulthood

The importance of neuropilins function and roles during embryogenesis has been also assessed through the generation of mice lacking one or the other neuropilin receptor. According to Kawasaki and collaborator, NRP1 null mutant embryos showed severe phenotype of various vascular defects resulting in death at E13.5 (Kawasaki et al., 1999). Additionally, NRP1 transgenic and deficient mouse models established an essential role of NRP1 in the development of the embryonic nervous and cardiovascular systems (Pellet-Many et al., 2008). In contrast neuropilin-2 knockout mice are viable but are characterized by abnormal lymphatic vessels including an abnormal patterning and marked reduction in small lymphatic vessels and capillaries (Yuan et al., 2002). Interestingly, double knock out leads to the most severe phenotype causing an earliest death at E8.5 exhibiting severe vascular defect (Takashima et al., 2002). Neuropilin co-receptors null mice exhibit interesting phenotypes; I will not review all null mutants here but will expose for example the plexin-A subfamily, which is associated with human breast tumorigenesis (Gabrovska et al., 2011). Interestingly, plexin-A1 null mice exhibit guidance defects of spinal commissural projections (Nawabi et al., 2010). Plexin-A3 knockout mice propose that plexin-A3 regulates the development of hippocampal axonal projections (Cheng et al., 2001). According to Suto and collaborators, plexin-A2 deficiency causes a shift of mossy fibers from the supra-pyramidal region to the infra and intra-pyramidal regions and plexin-A4 deficiency induces inappropriate spreading of mossy fibers (Suto et al., 2005). Concerning VEGFR null mutant mice, all are lethal and relate to vasculature disorders, indeed VEGFR-1 is lethal in mice at embryonic day E8.5 due to severe malformation of the vasculature (Fong et al., 1995). VEGFR-2 null mice are embryonic lethal between day E8.5-9.5. These animals have severe defects in endothelial and hematopoietic cell development with no organized blood vessel found at any point within the developing embryo (Shalaby et al., 1995). VEGFR-3 null mice are also embryonic lethal at day E9.5 and display cardiovascular failure as a result of the abnormal structure and organization of large vessels that leads to defective vessel lumens and an accumulation of fluid within the pericardial cavity (Dumont et al., 1998).

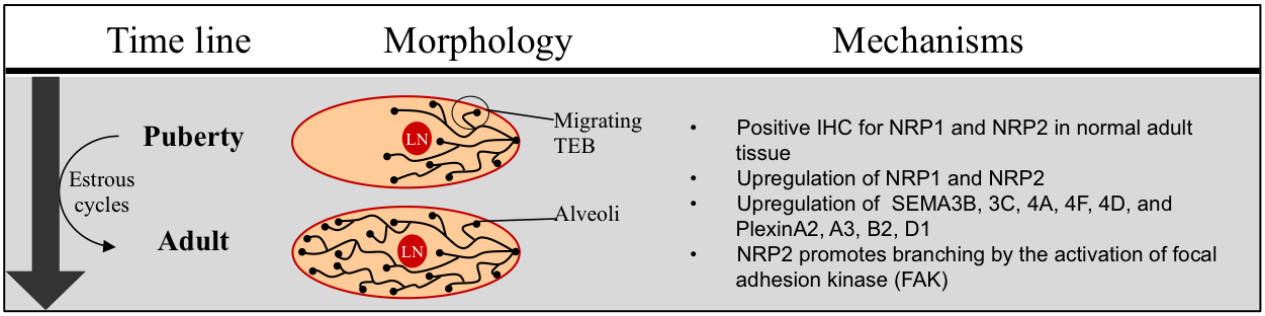


Figure 13: Implication of neuropilins network in the mammary gland

In brief, neuropilin-1 is essentialy involved in early vasculogenesis and neuropiline-2 is important for lymphangiogenesis during embryogenesis. Remarkably, neuropilin-1 is preferentially expressed in arteries while neuropilin-2 is rather stated in veins and in lymphatic vessels (Grandclement et al., 2011b).

In contrast with HER2 receptor, neuropilins harbor a wide pattern of expression in adults in normal and non-vascular tissues suggesting a pleiotropic role for these receptors in humans. The functional role of neuropilins, other than in the nervous system and vasculature, is not well defined and need further investigation. Besides neurons and endothelial cells, neuropilins are expressed by a variety of cells including dendritic cells, T cells, epithelial cells, platelets, adipocytes and tumor cells (Bielenberg et al., 2006; Ellis, 2006; Roth et al., 2008).

II.2.3) Neuropilins in the mammary gland

Very few studies focused on the functions of neuropilins in the mammary gland development and biology. Nevertheless, neuropilin-1 and neuropilin-2 immunohistochemistry have been assessed in the normal breast tissue. According to Stephenson et al., the myoephithelial cells of the mammary ducts and lobules are positive for NRP1 staining. In contrast, the inner epithelia cells were not positive to NRP1 staining. Intriguingly, only vascular smooth vessels of blood vessels from normal breast tissue were positive to NRP1 staining and not endothelial cells (Stephenson et al., 2002). Such as neuropilin-1, normal breast tissue expresses neuropilin-2 but the expression pattern is slightly different. Neuropilin-2 staining is spotted in blood and lymphatic vessels but not in normal breast epithelium (Yasuoka et al., 2009).

As mentioned above in mammary gland development a crucial step during puberty is the formation of the terminal end buts (TEB) within the mammary fat (Fantozzi and Christofori, 2006). These TEB appear at the tip of the ducts (figure 13) and will represent the invading front of these. In parallel, side branching and proliferation increase importantly (Watson and Khaled, 2008). TEB were isolated from surrounding tissues and gene expression profile of this isolate was compared with a normal duct. Numerous genes were upregulated in the TEB compared to the normal duct including SEMA3B, -3C, -4A, -4F and -4D, but also semaphorin receptors such as plexin-A2, -A3, -B2, -D1 and neuropilin-1 and -2 (Morris et al., 2006). Neuropilin-2 role in breast

development has been assessed with a transgenic mouse harboring a mammary gland specific knockout. This study reveals that neuropilin-2 promoted branching morphogenesis in the mammary gland by the activation of focal adhesion kinase (FAK), a protein clearly implicated in mammary gland branching and development (Goel et al., 2011). Taken together these findings suggest a potential role of the neuropilin platform signaling in ductal growth and morphogenesis in the developing mammary gland.

II.2.4) Neuropilins regulation

To date no specific study tackles the regulation of neuropilins specifically in breast cancer, however numerous studies have been conducted in endothelial cells and other types of cancers suggesting tumor necrosis factor alpha (TNFα), EGF and various transcription factors as potential modulators of neuropilins. Indeed in human umbilical vein endothelial cells (HUVECs), TNF-alpha treatment increases NRP1 expression but not NRP2 according to Yang and collaborators (Yang et al., 2004). Intriguingly, in the same cells, Giraudo observed adverse effect using this cytokine rather decreasing neuropilin-1 expression (Giraudo et al., 1998). EGF is also incriminated in neuropilin-1 upregulation in numerous human carcinomas including gastric cancer, pancreatic cancer and colon cancer (Akagi et al., 2003; Parikh et al., 2003, 2004). Transcription factors such as prospero homeobox protein 1 (prox-1) and the chicken ovalbumin upstream promoter transcription factor 2 (COUP-TFII), both involved in development, decrease NRP1 expression. Adverse result is sensed with the heart and neural crest derivatives-expressed protein, playing an essential role in cardiac morphogenesis, inducing an increase of NRP1 (Yamagishi et al., 2000; Hong et al., 2002; You et al., 2005). Notably, NRP2 expression is differentially regulated, indeed according to Bielenberg, prox-1 induces NRP2 while suppressing NRP1 and epidermal growth factor (EGF) induces NRP1 and not NRP2 (Bielenberg et al., 2006). Besides protein and transcription factors both neuropilins levels are increased following nerve transection or crush injury (Scarlato et al., 2003) and this is also appreciated after cerebral ischemia due to occlusion of the cerebral artery (Fujita et al., 2001).

Name	Description	Structure	Species
NRP1	Neuropilin-1	Full-lenght	Human
S11NRP1	Soluble neuropilin-1 isoform truncated at exon 11	Extracellular NRP1	Human
S12NRP1	Soluble neuropilin-1 isoform truncated at exon 12	Extracellular NRP1	Human
SIIINRP1	Soluble neuropilin-1 isoform truncated at exon 9 followed by exon 12	Extracellular NRP1	Human
SIVNRP1	Soluble neuropilin-1 isoform truncated at exon 10 followed by exon 12	Extracellular NRP1	Human
NRP2a(17)	Full-length neuropilin-2 added of 17 amino acids after amino acid 809	Full-lenght + 17aa	Human
NRP2a(22)	Full-length neuropilin-2 added of 22 amino acids after amino acid 809	Full-lenght +22aa	Human
NRP2b(0)	Full-length neuropilin-2	Full-lenght	Human
NRP2b(5)	Full-length neuropilin-2 added of 5 amino acids after amino acid 808	Full-lenght +5aa	Human
NRP2a(0)	-	-	Mouse
NRP2a(5)	-	-	Mouse
S9NRP2	Soluble truncated neuropilin-2 isoform	Extracellular NRP2	Human

Table 10: Nomenclature of neuropilin-1 and neuropilin-2 human and rodent homologue variants

II.2.5) Spliced variants

NRP1 and NRP2 are encoded by genes harboring 17 exons and 16 introns (Rossignol et al., 2000a). Concerning NRP1, up to date five spliced variant comprising one transmembrane and four soluble forms have been identified and characterized (table 10). The soluble forms include $_{S11}$NRP1 which is truncated at exon 11, $_{S12}$NRP1 which is truncated at exon 12, $_{SIII}$NRP1 harboring 9 exons and $_{SIV}$NRP1 possessing 10 exons both added of exon 12 (Gagnon et al., 2000; Rossignol et al., 2000b; Cackowski et al., 2004). These isoforms have conserved their extracellular domains responsible for ligand binding but c-domain, TM and intracellular domains are lacking. Such as HER2 soluble spliced variants, the soluble NRP1 isoforms are speculated to act as decoy receptors that bind and sequester the ligands thereby harboring a "protective" profile. Indeed Gagnon and collaborators reported that $_{S12}$NRP1 is able to provoke the tumor cell apoptosis by antagonizing VEGFR binding (Gagnon et al., 2000). Importantly, one study reports the importance of soluble NRP1 variants in breast cancer. According to Cackowski and colleagues $_{SIII}$Nrp1 and $_{SIV}$Nrp1 inhibit VEGF-A165 binding *in vitro* in MDA-MB-231 breast cancer cell lines and suppress VEGF-A165-induced cell migration and proliferation (Cackowski et al., 2004).

In contrast to neuropilin-1, neuroplilin-2 exhibits alternative splice variants of the full-length and only one soluble form (table 10): $_{S9}$NRP2. Here again the soluble isoform conserves its extracellular domains responsible for ligand binding but c-domain, TM and intracellular domains are lacking. Full length NRP2 isoforms include the NRP2A, and NRP2B and further sub-isoforms were subsequently described by Rossignol and coworkers giving rise to NRP2a(17), NRP2a(22), NRPP2b(0) et NRP2b(5). These isoforms result of the insertion of 17 and 22 amino acids after amino acid 809 (located after the c domain) for NRP2A isoforms and insertion of 0 and 5 amino acids after amino acid 808 (located after the c domain) for NRP2B isoforms. In mice, two additional variants, NRP2a(0) and NRP2a(5), were also reported (Chen et al., 1997). NRP1 and NRP2A show 44% amino acid identity in entire regions (Chen et al., 1997; Nakamura and Goshima, 2002; Bagri et al., 2009). Specific studies implicating one neuropilin-2 isoforms rather than another in tumorigenesis has not yet been investigated.

II.2.6) Novel targets for breast cancer and metastatic breast cancer

Neuropilins have not been extensively studied in breast cancer but interestingly some work has been investigated in MDA-MB-231 and MCF7, cells of particular interest for my *in vitro* and *in vivo* studies. Indeed, Bachelder and coworkers demonstrated that neuropilins promotes survival of MDA-MB-231 cells *in vitro* since the suppression of VEGF expression induced apoptosis, having in mind that NRPs are the only VEGF receptors in these cells. In the same paper but on another cell line, expressing neuropilin-1 in MDA-MB-453 breast cancer cells, protected the cells from hypoxia-induced apoptosis while mock transfected cells were not. Although some studies have indicated a pro-tumorigenic role of NRPs in breast cancer, other reports suggest that NRPs play a more complex role. Lee et al. found that survival effects of VEGF in the breast carcinoma MDA-MB-231 and MCF-7 cell lines were unaffected by NRP1-knockdown (Lee et al., 2007). Further experiments with MDA-MB-231 cell line showed that sema3B inhibited tumor cell growth and induced apoptosis, an anti-tumoral effect that could be mediated through NRP1 (Castro-Rivera et al., 2004).

Besides a role of the neuropilin network in mediating pro or anti-apoptotic signals, numerous studies suggest a role in migration and metastasis. Interestingly such as HER2, neuropilin-2 is correlated with lymph node metastasis and CXCR4 expression in breast cancer, a chemokine regulating chemotaxis and chemoinvasion (Fernandis et al., 2004; Yasuoka et al., 2009). However, neuropilin-2 expression is able to inhibit tumor cell metastasis to lung in a model of murine mammary carcinoma cell line (66c14) (Caunt et al., 2008). The expression of sema3E in mammary adenocarcinoma cells induces lung colony formation *in vivo* (Casazza et al., 2010). Moreover, in a three-dimensional co-culture assay, sema3A and sema3C promoted the migration and proliferation of MCF7 cells (Nasarre et al., 2005). In vivo expression of sema3E in mammary adenocarcinoma cells induces the ability to form lung metastasis when using the tail vein injection model (Christensen et al., 2005). Another study reported that VEGF is a mandatory factor for MDA-MB-231 breast carcinoma cell invasion and that neuropilin-1 is essential for this function (Bachelder et al., 2002). Here again if some studies report a pro-migratory and a pro-metastasic role of neuropilin signaling platform some studies report opposite effects. Indeed sema3A or NRP1-knockdown using siRNA or inhibiting plexin-A1

enhanced breast carcinoma cell migration (Bachelder et al., 2003). Consistent with an anti-metastatic role of semaphorins in breast cancer, sema3F has been reported to inhibit MCF7 motility through NRP2 (Nasarre et al., 2005). A previous paper from Nasarre and collaborators in 2003 demonstrated that that sema3F inhibits cell attachment and spreading in the breast cancer cell line MCF7 and C100 cells via NRP1 and NRP2 respectively.

Reasons explaining such discrepancies may include the wide-range of receptor and/or ligand repertoire within a particular tumor type and the associated tumor microenvironment. This is actually not surprising because numerous studies exemplified a competing effect of ligands when binding to neuropilins. Indeed, semaphorins can act as attractive and repulsive guidance signals during the development of cortical projection (Bagnard et al., 1998). In tumorigenesis, semaphorins exhibit pro and anti-tumoral effects (Nasarre et al., 2005) and in breast cancer Bachelder and colleagues observed a competing effect of sema3A and VEGF for the binding on neuropilin1 receptor during chemotaxis assays of breast carcinoma cells (Bachelder et al., 2003). Therefore broader studies are necessary to dissect the complex interplay of all the possible factors modulating the neuropilin platform in breast tumor biology.

Interestingly, the importance of neuropilins in human clinics has also been investigated. Both proteins are expressed in human breast cancer biopsies (Yasuoka et al., 2009; Ghosh et al., 2008; Jubb et al., 2012). Neuropilin-2 expression is correlated with poor patients survival and with lymph node metastasis (Yasuoka et al., 2009). Additionally Kaplan-Meier survival analysis showed that high levels of VEGF, VEGFR-1, VEGFR-2, and neuropilin-1 were also all significantly associated with worse survival in breast cancer (Ghosh et al., 2008). Such as HER2, neuropilins expression have been detected in many other human cancer including prostate, bladder, stomach, kidney, pancreas, colon, skin, ovary, lung, melanoma, leukemia, osteosarcoma, glioblastoma and neuroblastoma (Bilenberg et al., 2007).

Taken together, *in vitro, in vivo* and clinical findings indicate a crucial role of neuropilins in breast tumor invasion and metastasis in addition to their involvement in tumor vascularisation. Neuropilins are therefore ideal new attractive targets in breast cancer and some studies have shown this to be already the case *in vitro* and *in vivo*. Indeed, Barr and co-workers

demonstrated in 2005 that a peptide targeting the VEGF165-binding site on NRP1 antagonizes the autocrine anti-apoptotic effects of VEGF on 4T1 and MDA-MB-231 breast carcinoma cells *in vitro*. Moreover, another group also described an heptapeptide inhibiting VEGF binding to NRP1 thereby reducing tumor volume, blood vessel density and endothelial cell area in an orthotopic mammary carcinoma model of MDA-MB-231 cells (Starzec et al., 2006).

Of course targeting neuropilins has been already investigated in other fields than breast tumorigenesis. Genentech has for example developed an anti-NRP1 antibody (MNRP1685A) undergoing clinical evaluation. Up to date two phase 1 studies were initiated: a first study started in September 2008, evaluating in a dose-escalation study, the safety and pharmacological properties of MNRP1685A in patients with locally advanced or metastatic solid tumors. A second study, started in august 2009, combined MNRP1685A with bevacizumab with or without paclitaxel in patients with locally advanced or metastatic solid tumors. Unfortunately no result or publications have been posted yet concerning the first study according to the service of the United States national institutes of health website. However, the existence of adverse effects of the antibody has been reported during the 2011 American Society of Clinical Oncology (ASCO) annual meeting. Acute infusion reactions, mainly rash and pruritus, were frequent but tolerable with premedication. Platelet reductions were frequent, transient and recoverable. A higher than expected rate of clinically significant proteinuria, resulting in hold or discontinuation of the drug, was also observed. Genentech has also developed an antibody targeting neuropilin-2 receptor, which harbors already promising results in pre-clinical studies (Caunt et al., 2008). Up to date no clinical trials have been investigated using anti-neuropilin-2.

When analyzing preclinical and/or clinical trials in breast cancer field, HER2 is an outstanding validated target and mounting evidences suggest neuropilins as novel attractive targets. As appreciated in my thesis, there exist a plethora of strategies to inhibit HER2 but much less to tackle NRP1/NRP2 signaling. All of these strategies are essentially inhibiting either the extracellular or the intracellular receptor domain. As highlighted earlier the actual trend is to develop novel candidates with larger spectrum of action, inhibiting signaling platforms rather than inhibiting a single target. Indeed, the work of our laboratory is devoted to the preclinical development of such therapeutic compounds. However, this is done by following an original

strategy: interfering with the transmembrane domains of cancer associated membrane receptors.

III) Terra incognita: Transmembrane domains (TMD) and the Peptidic strategy

It is becoming increasingly clear that protein interactions are far more extensive than originally appreciated. Indeed, mounting evidence concurs in the current idea that the transmembrane regions are important in receptor association and signaling, and not thought only to be a passive anchor of the receptor in the membrane.

III.1) Transmembrane domain

TMD are usually composed of 20 to 23 residues and while polar residues are rare, the hydrophobic residues are over-represented. Various geometric motifs govern TMD interactions and associations of helical TMD are very dynamic. It should be stress that most TMD of single spanning proteins are usually more conserved than the rest of the protein, and interestingly one specific side of the helix is preserved, highlighting the preservation of a potential interacting motif (Hubert et al., 2010).

III.1.1) Once upon a time: Glycophorin A (GPA)

The first study demonstrating that protein dimerization could be driven by specific interactions between transmembrane alpha helices was performed on the Glycophorin A (GPA) (Lemmon et al., 1992). This protein is present on human erythrocyte membrane and is important for the classification of blood group antigens (Chasis and Mohandas, 1992). The TM segment of GPA is composed mostly of hydrophobic residues (ITLIIFGVMAGVIGTILLISYGI), a hydrophobic composition that is typical of single span TM domains. GPA is the most extensively characterized protein presenting a TMD interaction. Numerous studies comprising mutagenesis studies (Lemmon et al., 1992), reporter gene dimerization (TOXCAT) (Russ and Engelman, 1999), FRET quantification (Fisher et al., 1999) and finally the nuclear magnetic resonance (NMR) description structure of the TMD of GPA (MacKenzie et al., 1997) lead to the elucidation of a crucial motif controlling the TMD dimerization of this protein: the GxxxG motif (where x represents any amino acids and G are glycines)(Senes et al., 2000). This motif is characterized now in numerous other interacting transmembrane domains including TMDs of HER2 and neuropilins.

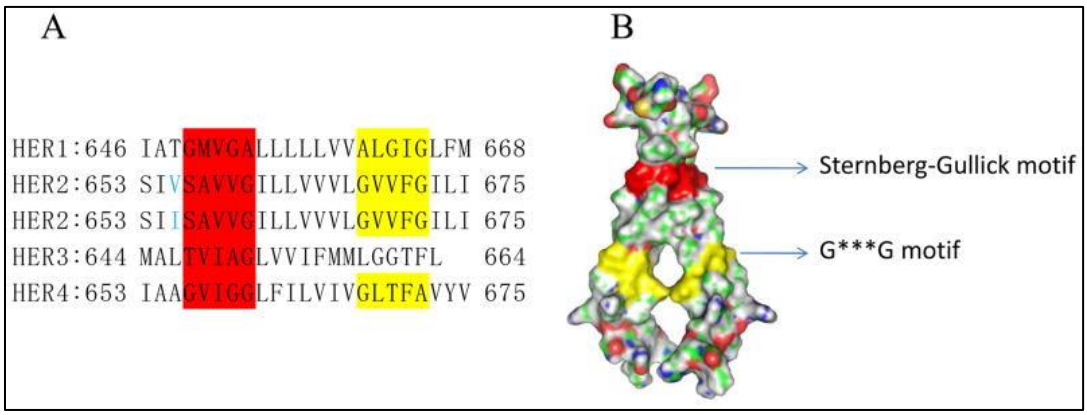


Figure 14: Sequences and structure of the human members of the EGFR family transmembrane domain. A) Dimerization motifs, in red sequences initially described by Sternberg and Gullick in 1990 and in yellow the GxxxG motifs. B) The surface of the HER2 transmembrane domain (Russ &Engelmann, 2000).

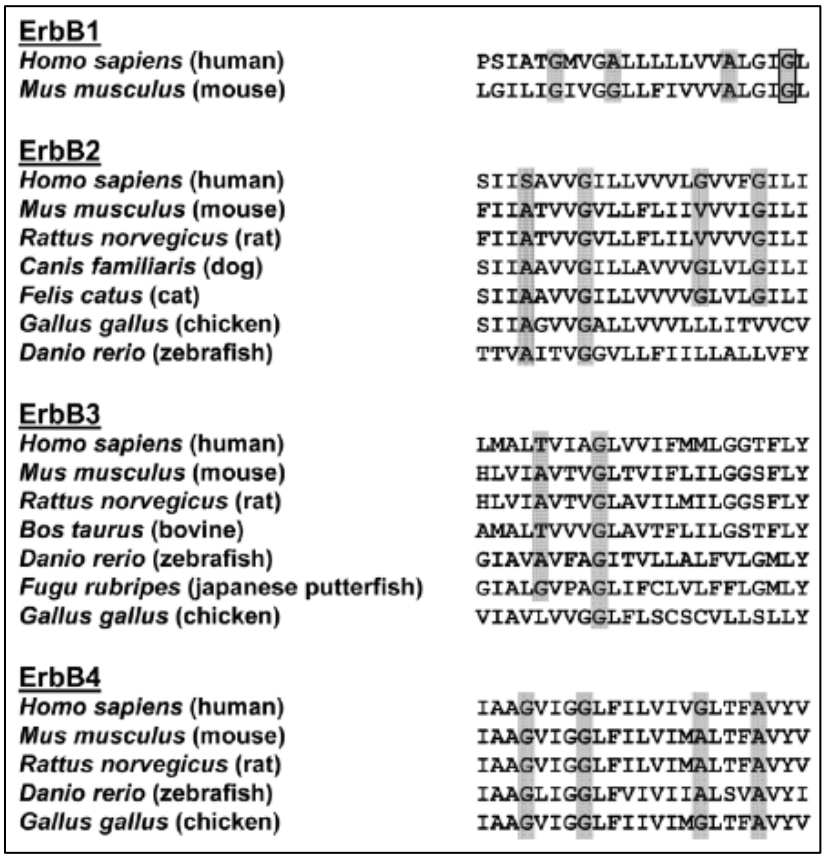


Figure 15: Alignment of ErbB receptors TMD sequences from different species (Cymer and Schneider, 2010)

III.1.2) Transmembrane domain of HER2

HER2 membrane-spanning alpha-helix domain is composed of 23 amino acids. Sequence alignment of TMDs of the members of the EGFR family denotes the existence of two dimerization motifs: the Sternberg-Gullick motif (Small aa-xxx-Small aa) and the GxxxG motif. As observed below, with the exception of HER3 lacking the GxxxG motif, all the members of the HER family display both motifs distant by 7 amino acids, assigning these on the same side of the alpha helix (Chothia et al., 1981)(figure 14). The isolated TMD of ErbBs can form homo- and heterodimers. According to Mendrola and collaborators, the TM domain of HER2 may undergo dimerization via either one of the two dimerization motifs (Mendrola et al., 2002). Intriguingly while both motifs are important for HER2 homodimerization, for EGFR only the C-terminal GxxxG motif (towards the intracellular space) would be implicated in the homodimerisation and the Sternberg-Gullick motif in the N-terminal region (towards the extracellular space) would rather participate in the heterodimerization of the EGFR with the HER2 receptor (Gerber et al., 2004). Interestingly, the N-terminal motif of the ErbBs was found to mediate and stabilize a slightly stronger interaction than the C-terminal motif (Cymer and Schneider, 2010). The driving forces between the dimerization motifs could be engendered by Van der Waals forces and hydrogen bonds between amino acids of each alpha helix (Bazley and Gullick, 2005). Noteworthy, homodimerization of ErbB receptor outer membrane domain has been extensively observed whereas heterodimerization of extracellular ErbB domains is barely undetectable (Ferguson et al., 2000). Thus suggesting the contribution of other receptor domain, such a TMD, in this dimerization process.

As mentioned above, they are two conformations of the extracellular domain, a closed inhibited or an open active conformation. This is completely consistent with computational mapping of the TM domain of ErbB2 receptor describing two stable conformations of the TM domain (Fleishman et al., 2002). They suggest that these conformations correspond to the active and inactive states of erbB2 and that the switch between one and the other interacting domain is very probable as the energy barrier between these two states is relatively low.

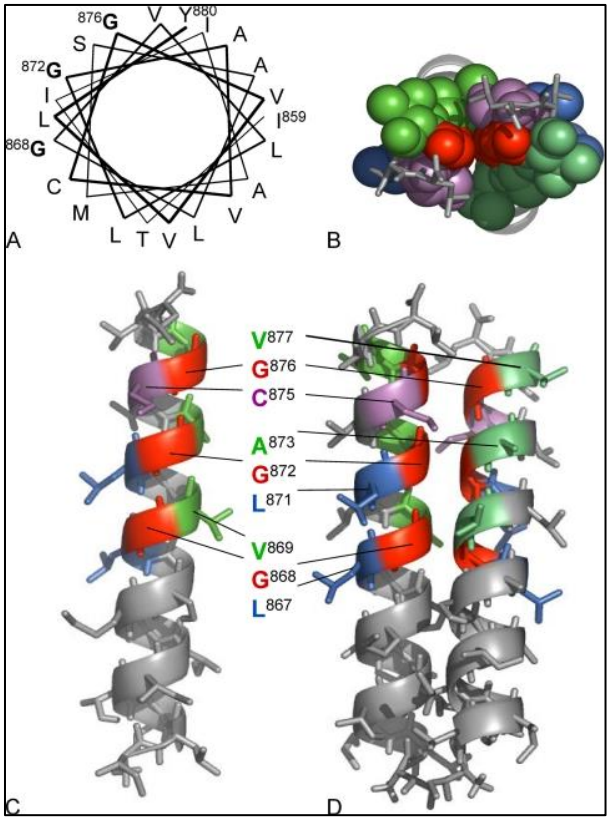


Figure 16: Predicted structural features of the NRP1 TMD. A) Helical-wheel representation of the dimer interface in the NRP1 TM domain, (B–D) possible structure of the NRP1 TM domain homo-dimer (Roth et al., 2008).

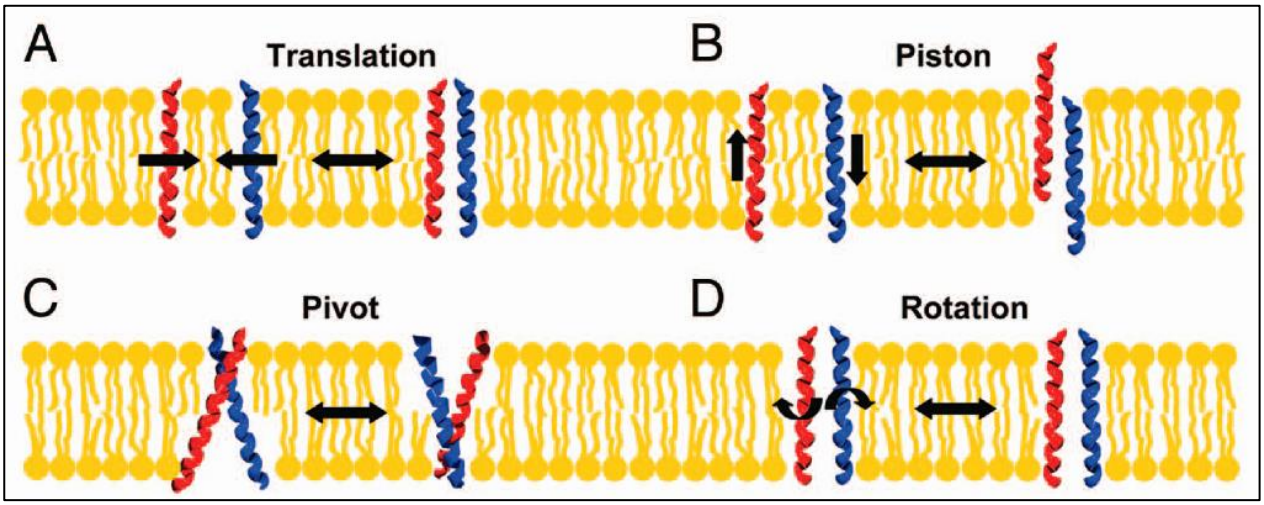


Figure 17: Principles of TM signaling. (A) Translational motion (B) Piston motion (C) Pivot motion (D) Rotation motion (Cymer and Schneider, 2010)

GxxxG-like interaction motifs appear to be well conserved in ErbBs TM helices among species (figure 15) and the distance with the Sternberg-Gullick is always conserved suggesting that motifs and their positioning are important for ErbBs activity.

III.1.3) Transmembrane domain of neuropilins

In contrast to HER2 TMD, less is known about the neuropilins transmembrane domains. Neuropilins have a transmembrane domain composed of 24 amino acid residues. Neuropilins TMDs also contain a putative dimerization GxxxG motif. Interestingly, the complete transmembrane domain is highly conserved; indeed it shares 100% amino acid identity across species (Takagi et al., 1995; Kawakami et al., 1996). In neuropilin-1 TMD the GxxxG motif, which is in fact a GxxxGxxxG motif is important for dimerization, oligomerization and is a prerequisite for sema-3A signaling (Roth et al., 2008) (figure 16).

III.1.4) Principles of TM signaling and dimerization motifs

Association between helical TM domains involves changes in the topography of TMD in a very dynamic manner. Four different types of movements of TMD within the membrane bilayer have been described (figure 17): 1) lateral translation in which the alpha helices move in the membrane plane, 2) piston motion were TMD move perpendicularly with respect to the bilayer, 3) in pivot motion the helices can move via a change in the crossing angle of the TM helices and finally 4) the rotation around the helix axis, resulting in a change of the orientation of the intra and extracellular domains in opposite membrane directions, is called the rotation motion. These TMD movements are supposed to be connected to signal transduction in conjunction with the extra or intra cellular domains.

Several motifs have been found to be involved in the association process of TMDs. The most common motif for interaction of two TMD helices was the one found in the glycophorin A (GPA) TMD: the GxxxG motif. The helix containing this motif can come in close proximity to each other, promoting further interactions between other residues. This motif is not sufficient for dimerization and surrounding residues are also of importance, this is observed in the MCP protein (Major Coat protein) where the extended motif LxxxGxxxGxxxT is participating to the dimerization (Melnyk et al., 2004). Motifs formed by the recurrence of GxxxG sequence are

called Glycin zipper motifs. Besides glycines, other small amino acids can also mediate helix-helix interactions. This is the case of the SmxxxSm motif, where “Sm” is a small residue (Gly, Ala, Ser or Thr), often referred to as a “GxxxG-like motif”, was proposed as a more general interaction motif for TM helices. Aquaporin-1 exhibits numerous SmxxxSm motifs that pack closely against one another suggesting the involvement of this motif in lateral interaction, but noteworthy some motifs are not involved in the alpha helices interaction (Li et al., 2012). Indeed, according to Hubert and coworkers (2010), one third of GxxxG motifs are not interacting with another helix.

With analogy to glycine zipper motifs, leucine zipper consists of multiple leucine residues repeats. This motif is also involved in helices dimerization. One of the major adhesion receptors expressed on the surface of circulating platelets, the glycoprotein Ib-IX-V complex harbors a leucine zipper motif in the dimerization interface (Wei et al., 2011). In the same way, a leucine zipper motif controls the dimerization of the transmembrane domain of the platelet-derived growth factorβ-receptor (PDGFβR)(Oates et al., 2010) such as in the erythropoietin receptor (EpoR)(Ruan et al., 2004).

Dimerizing motifs containing polar residues have also been described. The dimerization interface packs tightly the polar groups away from the hydrophobic bilayer allowing alpha helices to dimerize. A specific case is the QxxS motif, which has been found in the TMD of the bacterial aspartate receptor (TAR-1). Sal-Man and colleagues observed that polar to nonpolar mutation in the sequence reduced dimerization (Sal-Man et al., 2007). This motif has been extended to Polar-xx-Polar motif, in which polar amino acids include Ser, Thr, Glu, Gln, Asp, and Asn.

An aromatic-xx-aromatic motif has also been reported in transmembrane assembly. In the cholera toxin secretion protein EpsM, TM self-assembly is mediated by an aromatic motif (WxxW), here again dimerization was substantially affected by mutations in these specific positions (Sal-Man et al., 2007).

Another motif, the Ser/Thr rich sequences xxSSxxT and SxxxSSxxT can drive dimerization of transmembrane helices. Such motif is found in the TMD of Hepatitis C virus (HCV) non-structural

protein 4B, an integral membrane protein playing a role in the organization and function of the HCV replication complex (Dawson et al., 2002).

As more and more unique interacting helices are characterized, it is becoming clear that the sequence motif paradigm is incomplete. The existence of one or more of these short motifs does not necessarily imply a significant interaction, and both the adjacent residues (Dawson et al., 2002) and the location of the interactive surfaces (Johnson et al., 2006) also contribute positively or negatively to the interactions.

III.1.5) Importance of TMD for protein function

Studies reviewing mutation within TMD, using replacement of TMD or truncated receptors in tyrosine kinase receptor (TKR) highlight the importance of TMD in receptor association, function and signaling.

As described in figure 14, the position 655 in the human HER2 (blue) exhibits a Val/Ile single-nucleotide polymorphism. This Ile variant within the TMD of the HER2 receptor is linked to reduced risk of contracting breast cancer (Xie et al., 2000). Importantly, a point mutation within the transmembrane domain of the Neu receptor, the rodent homologue of HER2, resulting in a V664E mutated protein named NeuT (Bargmann et al., 1986) has been found to promote receptor dimerization and enhance tyrosine kinase activity of this ligand-less receptor (Weiner et al., 1989a). According to Sternberg and Gullick (1989) this activation can be explained by stereo-chemical considerations. The Glu in position 664 from one alpha helix (H1) will form a hydrogen bond with the Asp in the position 661 on the second alpha helix (H2). A second symmetric hydrogen bond will form between Asp661 of H1 and Glu664 of H2. Both hydrogen bonds account for the constitutive activation of this receptor. Noteworthy, this transforming Neu TMD has an importance in breast cancer, indeed transgenic mice harboring the Neu transforming sequence under the control of the mouse mammary tumor virus (MMTV) promoter lead to multifocal breast tumors in the mice mammary fat pads (Muller et al., 1988). In this mouse model the activated Neu oncogene appears to be sufficient to induce the malignant transformation in the mammary tissue. Other mutations present in the TMD of tyrosine kinases lead to the constitutive activation of the receptor. The mutation of the Val

Protein (OMIM ID)	Function	Disease(s)	Mutation(s)
I. Receptors			
erbB 2 (*164870)	RTK	Neuroblastoma (rat) Breast cancer polymorphism	V664E I655V
FGFR family	RTK	Dysplasias :	
FGFR1 (*136350)		Osteoglophonic dysplasia	C379R, etc.,
FGFR2 (*176943)		Beare-Stevenson Cutis gyrata syndrome	Y375C
FGFR3 (*134934)		Achondroplasia, Crouzon syndrome with Acanthosis nigricans	G380R, A391E, etc.,
FGFR4 (*134935)		Cancers	G 388R
IL-2 receptor beta chain (*146710)	Receptor for interleukin-2	Visceral leishmaniasis	G245R
II. Adhesion molecules			
Myelin Protein P0 (*159440)	Major structural protein of peripheral myelin	Charcot-Marie-Tooth disease, Dejerine-Sottas syndrome	I162M, G163R, G167R
TACI (*604907)	Tumor necrosis factor receptor	immunodeficiency	A181E

Table 11: Diseases associated with mutation in the TMD of single spanning proteins (nota bene: for FGFR4, “Cancers” are breast cancers)

residue in the position 938 to an Asp of the insulin receptor leads to its constitutive activation (Longo et al., 1992), in the same way the substitution of Val627 in a Gln leads to the constitutive activation of EGFR (Miloso et al., 1995). Moreover, some mutations in the TMD of single spanning proteins are disease associated. The mutation of Gly380 to a Arg in the fibroblast growth factor receptor 3 (FGFR3) TMD causes the most common genetic form of dwarfism, achondroplasia (Shiang et al., 1994). Other selected examples are found in the table 11 (Hubert et al., 2010).

Gardin and colleagues (1999) observed that the substitution of the insulin receptor transmembrane domain with that of glycophorin A lead in structural modifications that were unable to transmit the insulin signal properly. In contrast, numerous tyrosine kinase chimeric receptors in which the transformed Neu transmembrane domain replaced the native receptor TMD resulted in the constitute activation of the receptor. This was observed for the insulin receptor (Cheatham et al., 1993) and the PDGFR (Petti et al., 1998).

Tanner and Kyte (1999) also have shown that EGF-induced dimerization is far more efficient for a fragment of EGFR that contains both the extracellular and TM domains than the extracellular domain alone (Tanner and Kyte, 1999).

III.1.6) Strategies for studying TMD-TMD interaction within the cell membrane

I will not review here all known methods to study TMD-TMD interaction within the cell membrane but rather expose the bases of the most important strategies used in the context of my thesis.

III.1.6.1) Computational approaches: molecular dynamic modeling

Molecular modeling (MD) is assessed to describe the behavior of molecules and molecular system, and this could not be performed without a computer for extensive calculation. These methods provide a reasonably quick and efficient tool, as well as a quite rational atomic-scale model for the assessment of the mode of interaction of a dimeric structure in a membrane. A very simplistic view of the general process of molecular dynamic simulation can be described as the following four steps (Durrant and McCammon, 2011). Before starting a molecular dynamic

simulation, the computer model of the molecular system is prepared from X-rays structures, nuclear magnetic resonance (NMR), crystallographic or homology-modeling data. MD uses approximations based on Newtonian physics to simulate atomic motions. Then numerous equations and algorithms describing potential interactions of the atoms of the proteins of interest are estimated and parameterized, besides, chemical bonds and atomic angles are also modeled. In a third step, the positions of the atoms are moved according to Newton's laws of motion. As molecular dynamics simulations are computationally time consuming, it is only possible to calculate very short-term periods (such as a few nanoseconds up to microseconds). Finally, the simulation is assessed and the process is repeated several times. The contributions of the various atomic forces that govern molecular dynamics are called the “force field”. CHARMM (Brooks et al., 1983) and AMBER programs (Case et al., 2005) are validated force fields frequently used in the molecular modeling community. Membrane models of any degree of complexity can be used in MD calculations. Such as “force field”, validated membrane models are routinely used; these include di-myristoyl-phosphatidyl-choline (DMPC) and di-oleyl-phosphatidyl-choline (DOPC). According to Bocharov et al., in 2010, the influence of the membrane on the protein simulation can be assessed using 1) a full-atome hydrated bilayer, where protein interaction is considered in detail (that is each atoms of the protein), 2) the coarse-grain model, where groups of atoms (roughly four atoms) are packed together and replaced by a “grain” avoiding excessive computational calculation time and 3) a so-called hydrophobic slab membrane model, in which atomistic details of protein membrane interactions are not provided (Bocharov et al., 2010). A simulation will then generate a representative configuration of the mode of interaction of the dimeric structure of interest.

Besides molecular dynamics simulation, molecular docking and Monte Carlo approaches are also used. Ranking the different methods according to the amount of collected informations would place molecular dynamics in first position, followed by Monte Carlo approaches and finally the molecular docking method. Indeed using molecular docking technique, the membrane is either ignored or modeled basically and many physical factors of protein-protein and protein-lipid interactions are ignored but this technique allows a very quick scanning in comparison to the two other methods and therefore is used for an initial characterization. The

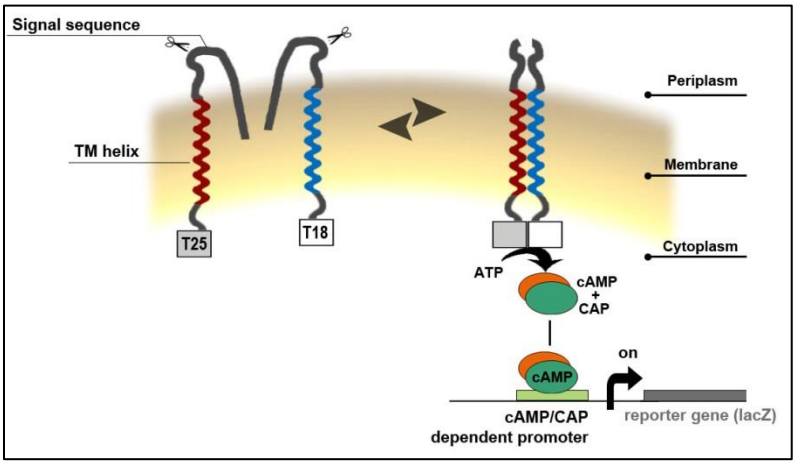


Figure 18: BACTH illustrated principle

Monte Carlo conformational search is more integrative than molecular docking approaches, membrane are more accurately taken into account. The most obvious difference with molecular dynamics is the dynamic parameter of interaction; MD provides wider information about the time-dependence of the properties of a molecular interaction (Bocharov et al., 2010).

The continuing progress in both computer power and algorithm design augur a bright future of computer-assisted drug design giving to molecular dynamics simulations a gradually important role.

III.1.6.2) Reporter assay: BACTH system

The BACTH assay or bacterial two-hybrid system is based on the recombination of adenylate cyclase (CyA) from Bordetella pertussis. This reporter gene assay relies on reconstitution of the catalytic domain of CyA which can be separated into two complementary fragments (Karimova et al., 2001), T25 and T28. When these two fragments are fused to interacting peptides, here the transmembrane domains of interest, if heterodimerization occurs, a functional adenylate cyclase enzyme unit can form. This is followed by the production of cyclic AMP (cAMP) in an E. coli strain, lacking its own adenylate cyclase. The synthesis of cyclic AMP is proportional to the association propensity of the two fragments and can be measured in a number of ways (Battesti and Bouveret, 2012). Dimerization leads to the reporter gene activation, here lac Z, and basically the stronger is the interaction between the TM domains, the higher is the β-galactosidase activity (figure 18).

BACTH method has already been assessed for various TMD of interest in the work frame of a collaborative project with the team of Dr P. Hubert in Marseille (Sawma et al. under revision). Original results are presented below in the result section. Besides BACTH, other reporter gene assays are currently used including TOXCAT and TOXluc assays. In the same way a reporter gene is activated after oligomerization of the transcription factor ToxR occurring only after TMD dimerization (Roth et al., 2008; Russ and Engelman, 1999).

III.1.6.3) FRET: Förster Resonance Energy Transfer measurements

Another potent method to study interactions between protein partners involved in a typical biomolecular process is FRET. This technique offers the possibility to evaluate the dimerization

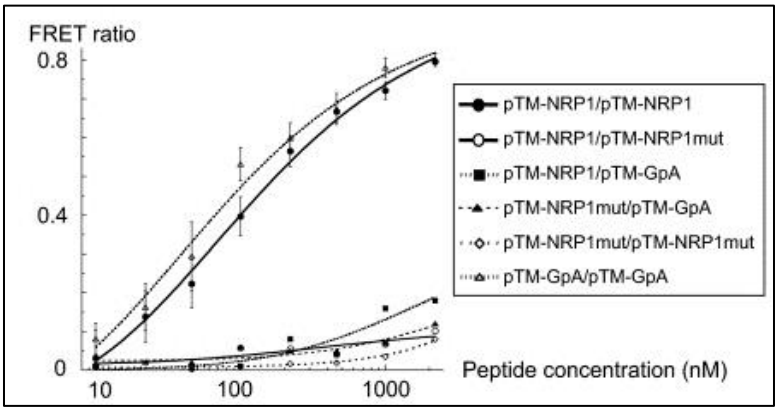


Figure 19: FRET ratio of various TMD including NRP1 and GPA (Roth et al., 2008)

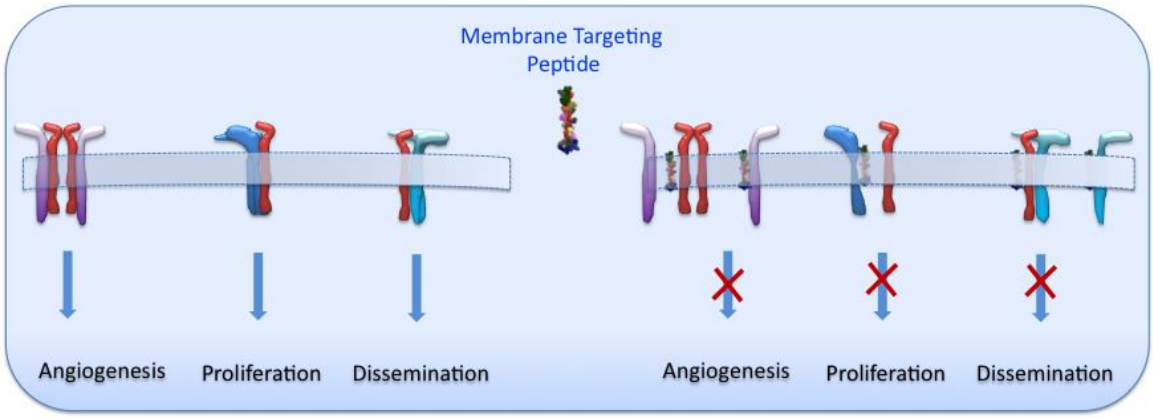


Figure 20: Peptidic strategy

capabilities between two molecules within several nanometers, a distance sufficiently close for molecular interactions to occur. Fluorescence resonance energy transfer involves a radiationless transfer of energy occurring from an excited state fluorophore, so called fluorescent donor, to a second fluorophore, the fluorescent acceptor, in close proximity. Thus if the two fluorophores are close enough, such as in a TMD interacting dimers, FRET will occur. Typical donor/acceptor pairs are commonly used, for example pyrene/coumarin, Cy3/Cy5, and EGFP/Cy3 (Wouters et al., 2001; Demchenko, 2010). This technique offers measurement with high sensitivity, specificity, rapidity and simplicity (Clegg, 1995). FRET analyses have already been achieved for the TMD of the neuropilin-1 dimer (Roth et al., 2008) (figure 19).

III.2) Interfering peptide strategy

III.2.1) Hypothesis

The role of TMD has changed dramatically the past 10 years, once mostly considered as a membrane anchor, it is now recognized as full-time actors in protein-protein interactions. As interaction between TMD occurs during the dimerization of the full-length receptors, the introduction in the membrane of homologous sequences mimicking the TMD should act as competitors of the dimerization and therefore inhibit downstream signaling. In this way, specific receptors might be targeted for inhibition using small peptides inserted in the membrane bilayer (figure 20).

III.2.2) A prophetic view slow to materialize

Initially, Lofts and coworkers demonstrated that a plasmid encoding mutated TM sequence of the rat Neu receptor could inhibit cell growth and tumor in nude mice (Lofts et al., 1993). Then, in a similar experiment using expression vector encoding for peptides of the native and of mutated TMD of ErbB2, Bennasroune and coworkers observed a specific inhibition of the autophosphorylation and signaling of the ErbB2 receptor confirming Lofts's work in 1993 (Bennasroune et al., 2004). This block in signaling involved inhibition of dimerization. Using the same expressing vector strategy, peptides mimicking the EGFR and the insulin TMD receptor inhibited the transphosphorylation and signaling of both receptors (Bennasroune et al., 2004, 2005). Again, similar results were observed in another study in which the TM domain of the

mutated Neu specifically inhibits the phosphorylation of the full-length mutated Neu *in vitro* (He et al., 2011).

Using differential epitope tagging, Hebert and colleagues in 1996 observed that beta2-adrenergic receptors form homodimers in which transmembrane domain VI of the receptor may represent part of an interface for receptor dimerization (Hebert et al., 1996). Noteworthy, a peptide derived from this domain, that inhibits dimerization, also inhibits beta-adrenergic agonist-promoted stimulation of adenylyl cyclase activity. Using the same approach for another G-coupled receptor (GPCR), the D1 dopamine receptor (D1DR), a peptide based on the TMD VI was able to specifically inhibit D1DR binding and function but without affecting receptor oligomerization (George et al., 1998). In contrast, on the dopamine receptor D2, peptides derived from the amino acid sequence of the TMD region VI affected the dimerization of the receptor and subsequent function (Ng et al., 1996).

A synthetic peptide derived from the transmembrane sequence of the T-cell receptor (TCR) called the core peptide (CP) exhibited immune-inhibitory effect as an anti-inflammatory peptide by inhibiting B and natural killer cell function (Huynh et al., 2003). In numerous *in vivo* studies, the core peptide (GLRILLLKV) reduced the T-cell mediated inflammation (in animal models of arthritis, allergic encephalomyelitis and diabetes mellitus) (Manolios et al., 2010) and in T-cell mediated skin diseases (Göllner et al., 2000). To investigate the effects of this peptide in humans, patients with psoriasis, atopic eczema, or contact dermatitis were treated topically with the core peptide. Most of the patients showed a marked improvement or full cured of their skin disease (Göllner et al., 2000).

In a different study, Yin et al in 2006 showed that an exogenous peptide that corresponds to the TMD of the integrin $\alpha 2\ \beta 3$ is capable to interact with the TMD of the native protein and is able to activate these integrins *in vitro*. Here the activated state of integrins is completed when alpha and beta chains are dissociated, these two chains being heterodimerized at the resting state (Yin et al., 2006).

TM peptides have also been studied as potential therapeutic agents for infections. Indeed encouraging results were observed treating lymphocytes with TM peptides in the context of

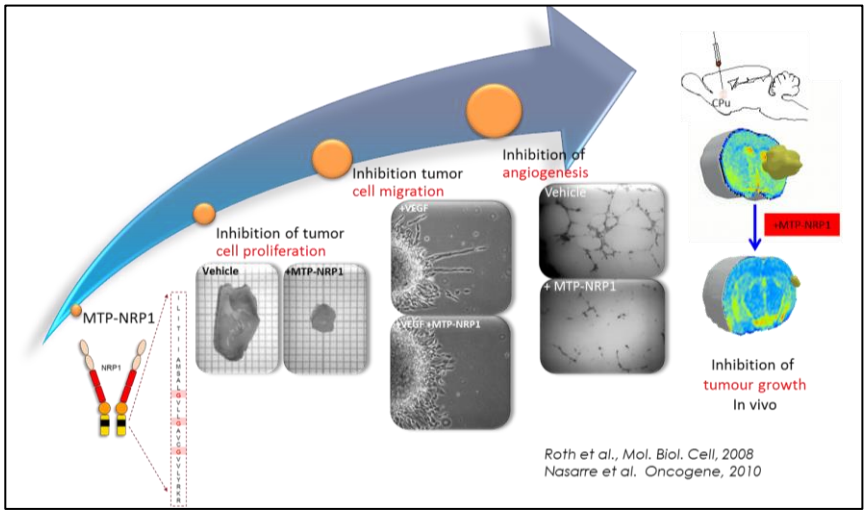


Figure 21: Preclinical validation of MTP-NRP1 for glioblastoma treatment

HIV-1 infection. These peptides were able to inhibit simultaneously the replication and the infectivity of HIV-1 (Manolios et al., 2010).

Remarkably, in my lab preclinical validation of the use of a peptide targeting the TMD of neuropilin-1 has been validated. Roth and coworkers observed in 2008 that this synthetic peptide abolished the inhibitory effect of Sema3A and mutation studies of the GxxxG dimerization motif in the TMD of NRP1 confirmed its biological importance for Sema3A signaling. In further experiments MTP-NRP1 (membrane targeting peptide-NRP1) exhibited both *in vivo* and *in vitro* anti-proliferative, anti-migratory and anti-angiogenic properties combating brain tumor growth (Nasarre et al., 2010)(figure 21).

Hence, interfering with TMD is becoming an unexpected therapeutic option in a wide range of fields ranging from immunology, cardiology, virology, to diseases such as cancer. Importantly the peptide-based interference of TMD has already been achieved in clinics with the example of the core peptide. Facing the resistance to conventional drugs in many cancers, exploring how to translate TMD interfering peptides into anti-cancer drugs is obviously a timely challenge.

IV) Thesis objectives

When I started my PhD in October 2010, tremendous work had already been achieved in my lab on the peptidic strategy (Bennasourne et al., 2004; Roth et al., 2008; Nasarre et al., 2010). However, little was known on the validation of this strategy on a confirmed therapeutic target ErbB2 in an *in vivo* breast cancer model. Moreover at that time, very little if anything was known on the potential of inhibiting neuropilin-1 and neuropilin-2 in breast cancer with such strategy. I was therefore in charge of the preclinical validation of an arsenal of three peptides: MTP-ErbB2, MTP-NRP1, MTP-NRP2 in the breast cancer context. At the same time, as breast cancer cells inflict their lethal effect when they metastasize to distant organs, it was also of interest to examine whether these peptides had an efficacy on breast cancer metastases.

In this context I had two main objectives:

- To evaluate the potential of the MTP-ErbB2 peptide to antagonize ErbB2, a validated target in breast tumor growth and metastasis, and to elucidate subsequent intracellular mechanisms.

- To assess whether targeting neuropilin-1 and -2 with membrane targeting peptides would impede breast tumor growth and metastasis.

To fulfill these objectives, I developed a similar workflow to evaluate the efficacy of the three transmembrane targeting peptides. First step consisted in a functional validation of the peptides efficacy *in vitro* while second step tested peptides in relevant *in vivo* breast cancer models. While *in vitro* assays were routinely assessed in the lab, I had to establish and validate all the *in vivo* models for drug potential evaluation. In parallel, an important issue during my PhD was to monitor closely the breast tumor growth and its related metastasis. This is the reason why different imaging methods were used and adapted to the various *in vivo* models I established.

MTP-ErbB2 characterization using the BACTH system or computational models work was done in a collaborative framework with the group of Dr Pierre Hubert (Marseille) and Dr Monique

Genest (Orléans) who performed the experiments respectively. Then, we investigated the impact of the peptide on ErbB2 signaling *in vitro* (by adding the peptide in the conditioned medium), confirming results previously obtained in my lab using expression vector encoding for peptides of the native and of mutated TMD of ErbB2 (Bennasroune et al., 2004). Finally, *in vitro* observations were confirmed *in vivo* using a spontaneous model of breast cancer: the MMTV-NeuNT mouse model. Besides confirming the inhibition of HER2 signaling *in vivo*, the peptide increased overall survival compared to control animals. Here the peptidic strategy, directed against a validated therapeutical target, has been for the first time applied in an *in vivo* breast cancer model. I was able to monitor breast cancer metastases occurrence and growth over time thanks to the imaging platform in IPHC (Institut pluridisciplinaire Hubert Curien) including a microCT. Upon 3D reconstruction of lung metastasis I was able to evaluate the impact of the peptide treatment on breast cancer metastasis.

For the assessment of the functionality of MTP-NRP1 and MTP-NRP2 peptides, I completed different *in vitro* models including proliferation assays. As triple negative breast cancers have still no targeted therapy and reveal very bad prognosis, proliferation assays were assessed on MDA-MB-231 cells (ER-,PR-,HER2-) harboring a triple negative phenotype. In this first step I was able to demonstrate for the first time that MTP-NRP1 receptors could be targeted in the triple negative breast cancer context. To confirm MTP-NRP1 and to evaluate MTP-NRP2 anti-angiogenic properties I used an *in vitro* tube formation assay, and an *in vivo* mouse retinal angiogenesis model. In both models, both drugs exhibited anti-angiogenic properties. To further evaluate the potential of both drugs *in vivo* we decided first to use an orthotopic breast cancer model to measure the impact of such strategy on the primary tumor growth. In a second step, as woman breast cancer metastasis related deaths remains still a sobering fact, we tested whether MTP-NRP1 and MTP-NRP2 would impede metastasis progression using a systemic breast cancer metastasis model. Here the monitoring of the primary tumor growth or the metastasis was assessed using luciferases cells and the NightOWL bioimager apparatus. I was able here again to prove the beneficial property of the elegant peptidic strategy directed against two new breast cancer targets: neuropilin-1 and neuropilin-2.

V) Material and Methods

In this section one will find methods for which further details needed to be added (such as for the MMTV-NeuNT mouse model and the computed tomography (CT) imaging apparatus) as well as standard supplementary technical data.

V.1) MMTV-NeuNT mouse model

The neu gene was first identified in rat tumors that had been induced by the carcinogen ethyl nitrosourea. The gene induces synthesis of a tumor antigen with a relative molecular mass of 185 kDa, a protein closely homologous to, but distinct from, epidermal growth factor receptors. The protein product is composed by an extracellular putative binding domain, a transmembrane domain, and an intracytoplasmic tyrosine kinase domain (Shih et al., 1981; Schechter et al., 1984). As exposed earlier the activation of the Neu oncogene in these tumors occurs by the substitution of Val in position 664 to Glu in the transmembrane domain of the protein (Bargmann et al., 1986). The presence of this transmembrane mutation causes an increase in the tyrosine kinase activity of Neu (Bargmann and Weinberg, 1988; Stern et al., 1988; Weiner et al., 1989a) by inducing ligand-independent receptor oligomerization (Weiner et al., 1989b). For the sake of clarity wild type Neu will be noted NeuWT and mutant or activated Neu will be noted NeuNT. Transmembrane sequences are exposed below; in bold underlined the dimerization motifs:

Rattus norvegicus (rat) NeuWT	FII**A**TV**V****G**VLLFLIL**V**VVV**G**ILI
Rattus norvegicus (rat) NeuNT	FII**A**TV**E****G**VLLFLIL**V**VVV**G**ILI

According to Uniprot analysis (http://www.uniprot.org/), the NeuWT TMD shares 64% homology with human HER2 TMD, and 88% with the entire human protein sequence.

The mouse mammary tumor virus, formerly known as the Bittner virus, is an onco-RNA virus of the Retroviridae family (Callahan et al., 2012). Various MMTV promoters exist and among these, MMTV-LTR has most frequently been used to express a gene of interest in the mammary epithelium since the promoter is active in both non lactating and lactating females (Taneja et

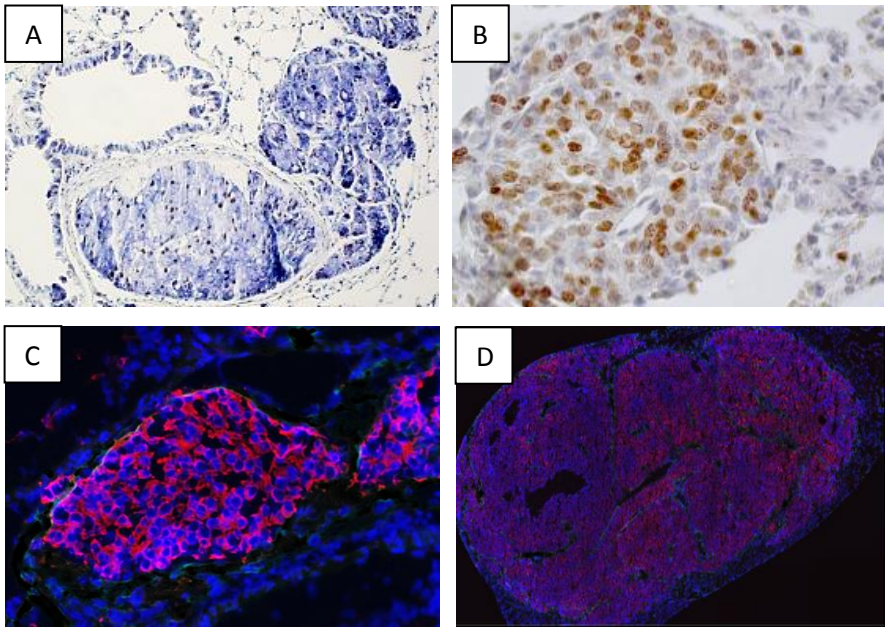


Figure 22: Picture of MMTV-NeuNT lung metastasis. (A) a “mixed” lung metastasis (intravascular and parenchymal), (B) Ki67 staining on a parenchymal lung metastasis, (C) CK8 staining on a parenchymal lung metastasis (Thesis Ines Velasquez 2013), (D) NeuNT staining on a parenchymal lung metastasis (Thesis Ines Velasquez 2013).

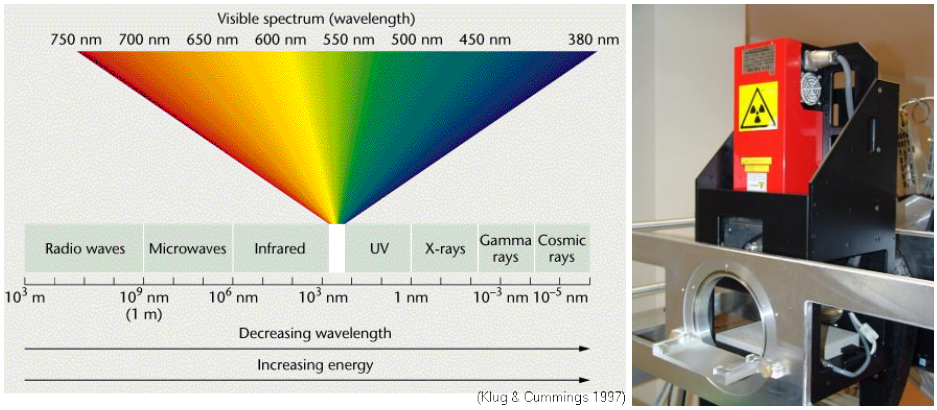


Figure 23: Electromagnetic spectrum and CT device

al., 2009a). Genetic background of transgenic mice is also of importance. Indeed, it is generally believed that the FVB/N strain is more susceptible to mammary tumors than mice of the C57BL/6 strain. For instance, FVB MMTV-NeuWT females develop mammary tumors at approximately 7 months of age, whereas FVB × C57BL/6 (F1) MMTV-NeuWT mice have tumor latencies greater than 18 months (Rowse et al., 1998).

An important issue in the MMTV-NeuNT model is the occurrence of lung metastasis. Even though metastasis occurrence is reaching 20% according to Muller and coworkers, this is clearly related to the time of observation of these events in the lungs. In my hands, 90% of MMTV-NeuNT transgenic mice displayed lung metastases at time of sacrifice (more or less 3 months). Interestingly, lung metastases occur through two distinct patterns: intravascular and/or parenchymal (Guy et al., 1992; Moody et al., 2002; Siegel et al., 1999). These lung metastasis are Neu positive, CK8 positive (Thesis Ines Velazquez, 2013) and Ki67 positive suggesting that these cells arise from the primary epithelial mammary tumor, as the lung parenchyma is Neu and Ki67 negative (figure 22). Mounting evidences suggest that lung metastasis arise from a group of cells (an emboli) from the primary mammary tumor (Kosanke et al., 2004; Sugino et al., 2002) and travel as such to the lungs. In this transgenic models emboli would then be arrested in the lung vasculature and eventually invade the lung parenchyma in a second step.

V.2) Imaging: Computerized tomography (CT)

X-rays were discovered in 1895 by Wilhelm Conrad Rontgen. An X-ray is an electromagnetic radiation characterized by a wavelength ranging from 0.01 to 10 nm and energies ranging from 100 eV to 100 KeV (figure 23).

Computed tomography was introduced in the early 1970s by Sir Godfrey Hounsfield and first applications were for neuro-radiobiology. A computerized tomography (CT) apparatus is composed of a gantry, the device housing the X-ray tube and detectors, with a large opening into which the patient or the animal is inserted.

The X-rays are produced in an X-ray tube which is composed of a cathode and an anode (also called target). Briefly, in the cathode, from the heating of a metal, electrons are produced.

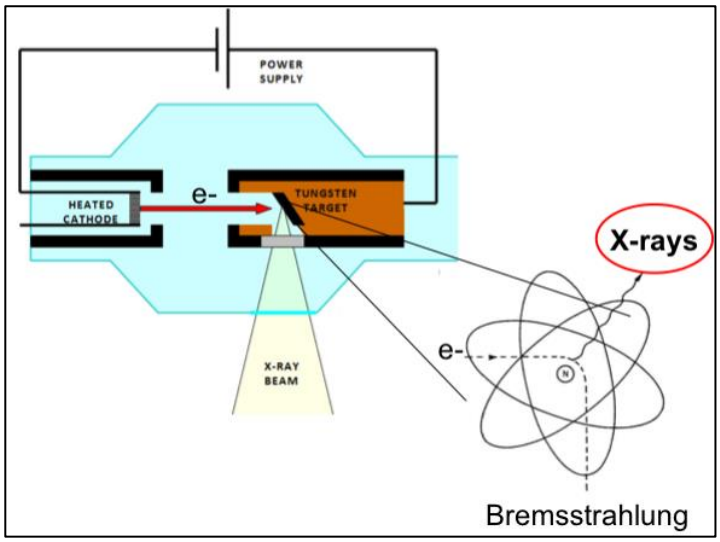


Figure 24: Production of X-rays in an X-ray tube

Tissue	CT Number (HU)
Bone	+1000
Liver	40-60
White mater	-20 to -30
Grey mater	-37 to -45
Blood	40
Muscle	10-40
Kidney	30
CSF	15
Water	**0**
Fat	-50 to -100
Air	-1000

Table 12: Hounsfield scale

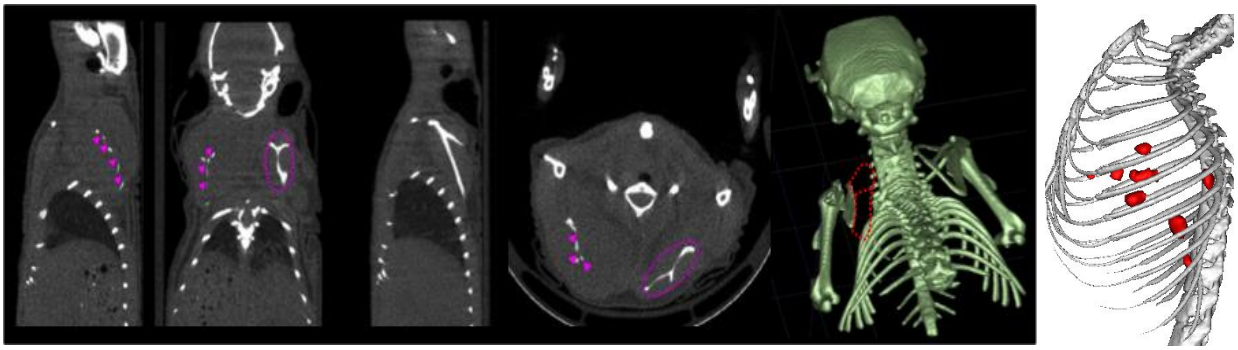


Figure 25: Computed tomography reconstructed images (2D and 3D)

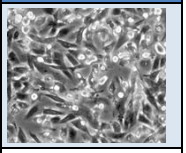
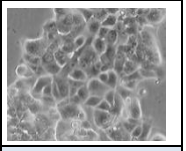
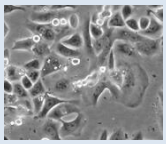
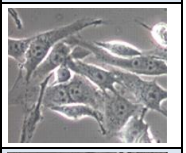
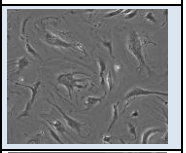
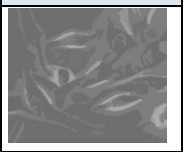
These are then accelerated and attracted into the anode (tungsten target) and once entered in the anode the electron will be decelerated. Indeed when these electrons will arrive next to the nucleus of an atom they will be deviated due to the positive charge of this particle attracting the electron. This deviation will decelerate and deflect the electrons and this energy change induces the emission of a photon in the X-ray range. This deceleration is called the bremsstrahlung effect (figure 24).

The remaining X-ray energy that has not been attenuated by the patient/animal, will be captured by the detector. This detector has to remain aligned with the X-ray source. CT images are obtained through the use of an X-ray tube and detector that rotate around the patient/animal which is stationary. The CT signal is measuring the average linear attenuation coefficient between the X-ray tube and the detector, that is the degree to which the X-ray intensity is reduced by a material according to the individual densities though which X-rays travel. The density information is transferred from the detector to the computer. Basically the greater the attenuation is, the brighter the pixel is (such as for bones), in contrast the less attenuation is, the darker the pixel will be as observed with air. The density values corresponds to a range of numbers evaluated thanks to the Hounsfield scale ranging from +1000 Hu, to -1000 Hu (Unit : Hu), calibrating universally air at -1000 Hu, water at 0 Hu and bone at +1000 Hu (table 12).

A sufficient number of transmission measurements is taken at different orientation of the X-ray source and detectors while rotating around the patient. By assigning different levels to different attenuation coefficient a 3D image can be reconstructed thanks to multiple calculation algorithms using a computer. The example in figure 25 shows a 3D reconstruction of a bone metastasis and lung metastases using the CT apparatus.

CT applications include detecting a wide range of abnormalities or diseases in any part of the body such as cancer, trauma, infection, inflammation, pneumonia, tuberculosis, angiography, stroke, bone fracture, sinusitis, spinal column damage, and much more.

V.3) Cell lines

Cells	Source	Origin	Morphology
MDA-MB-231	derived from an epithelial human breast adenocarcinoma (pleural effusion), ER-/PR-/HER2-	ATCC	
MCF7	derived from an epithelial human breast adenocarcinoma (pleural effusion), ER+/PR+/HER2+	Gift from Dr Patrick Nasarre	
SKBR3	derived from an epithelial human breast adenocarcinoma (pleural effusion), ER-/PR-/HER2++	Gift from Dr Pierre Hubert	
4T1	derived from an epithelial murin Balb/c spontaneous tumor, highly metatastatic (lungs, liver, bone, brain), Neu +	Gift from Dr Fanny Mann	
HUVEC	derived from human umbilical vein endothelial cells	Promo Cell	
NeuNT	derived from an epithelial mammary MMTV-NeuNT breast adenocarcinoma, Neu +	Home - derived	

MDA-MB-231, MCF7, SKBR3 and NeuNT cells were grown in Dulbecco's modified Eagle medium (DMEM, GIBCO), 4T1 cells were grown in Roswell Park Memorial Institute medium 1640 (RPMI, GIBCO) and HUVEC cells were cultured in Endothelial Cell Growth Medium (Promo Cell). All cell mediums were supplemented with 10% fetal calf serum (FCS) (GIBCO), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma), and cultured at 37ºC, 5% CO2. HUVEC cells were cultured with added supplements: ECGS (0.004ml/ml), hEGF (0.1ng/ml), hbFGF (1ng/ml) and FCS (0.02ml/ml). Cultures were grown to 70 to 80% confluence (not grown over 100% confluency) and were routinely split in 10 cm culture dishes. Culture were suspended with trypsin-EDTA (0.05% trypsine, 0.02% EDTA), spin down and split regularly up to 40 passages before newstocks were thawed with the exception of HUVEC cells split up to 5 passages.

First Objective

VI) To evaluate the potential of the MTP-ErbB2 peptide to antagonize ErbB2, a validated target in breast tumor growth and metastasis

Paper under revision

Transmembrane domain targeting peptide antagonizing ErbB2/Neu inhibits breast tumor growth and metastasis

Alexia Arpel[1,5], Paul Sawma[2#], Caroline Spenlé[1#], Norbert Garnier[3], Inés Velázquez-Quesada[1#], Thomas Hussenet[1#], Samia Aci-Sèche[3,4], Nadège Baumlin[1], Monique Genest[3], David Brasse[5], Pierre Hubert[2], Gérard Crémel[1], Gertraud Orend[1#], Patrice Laquerrière[5#] and Dominique Bagnard[1*]

[1]INSERM U 1109, Labex Medalis, Strasbourg University, Strasbourg 67200, France

[2]CNRS LISM UMR 7255, Aix Marseille University, Marseille 13402, France

[3]CNRS UPR 4301, Center for Molecular Biophysics, Orleans University, Orleans 45000, France

[4]ICOA UMR 7311, Orleans University, Orleans 45100, France

[5]CNRS UMR 7178, Institut Pluridisciplinaire Hubert Curien, Strasbourg University, Strasbourg 67037, France

[#]Equal contribution

[*]**Corresponding author:**

Dr Dominique Bagnard

INSERM U1109 Université de Strasbourg, Labex Medalis

3 avenue Molière, 67200 Strasbourg, France

Tel : +33-(0)3-88275364 Fax : +33-(0)3-88263538 Email: bagnard@unistra.fr

Running title: New anti-ErbB2 peptide inhibiting breast cancer

Total number of characters: 26813

Summary

Breast cancer is still a deadly disease despite major achievements in targeted therapies. Current targeted therapies have been designed to block ligand or ligand binding subunits for major tyrosine kinase receptors. But relapse is significant and metastases deleterious which demands novel strategies to fight this disease. Here we report a proof of concept experiment demonstrating that small peptides interfering with the transmembrane domain of the tyrosine kinase epidermal growth factor receptor ErbB2 exhibit anti-cancer properties when used at micro-molar dosages in a genetically engineered mouse model of breast cancer. A two hybrid-like assay served to demonstrate the specificity of the ErbB2 targeting peptide that induced long term reduction of ErbB2 phosphorylation and Akt pathway consistent with reduced tumor cell proliferation and survival. Micro-computed tomography analysis proved the anti-metastatic activity of the peptide also impacting primary tumor growth. This reveals the heart of the cell membrane as a novel dimension for drug design.

Introduction

Breast tumorigenesis is a multistep process leading cells to undergo genetic and epigenetic transformations eventually giving rise to invasive carcinoma with bad prognosis (Bombonati and Sgroi, 2011). The ErbB family of tyrosine kinase receptors plays a key role in breast carcinogenesis. One of the striking characteristics of this family is the existence of a complex signaling network made of multiple heterodimeric combinations of ErbB receptors to ensure proper signaling of their ligands such as epidermal growth factor (EGF), transforming growth factor alpha (TGFα), EGF-like growth factors or the neuregulins (Eccles, 2011). While having no identified ligand, ErbB2 is amplified and over-expressed in high grade ductal carcinoma (Ross and Fletcher, 1999) and in high grade inflammatory breast cancer (Charafe-Jauffret et al., 2004) thereby being largely involved in the induction and development of the malignant transformation (Freudenberg et al., 2009). Several studies revealed the importance of the dimeric status of ErbB2 triggering signaling cascades including the MAPK and PI3K/Akt pathways (Freudenberg et al., 2009). ErbB2 promotes cell proliferation, supports survival and also favors invasion and metastasis. Most of the ErbB2-positive cancers resist to ErbB2-targeted therapies or tyrosine kinase inhibitors (Arteaga et al., 2012). The heterodimerization capacity of ErbB2 with ErbB1 or ErbB3 and activation of compensatory signaling pathways are considered as important resistance mechanisms (Yamaguchi et al., 2013). An ideal therapeutic strategy would hence be to interfere with receptor interactions by preventing dimerization and oligomerization in order to block redundant or compensatory downstream signaling pathways. Mounting evidence nowadays demonstrate that the transmembrane domains act as crucial regulators of integral membrane receptors interactions. Strikingly, the transmembrane domains of ErbB family members are also critically involved in ErbB signaling by not only stabilizing dimerization but also by controlling structural rearrangements favoring optimal conformational positioning for kinase activation (Cymer and

Schneider, 2010). Hence, point mutations in the transmembrane domain of the murine homologue of ErbB2 (mErbB2 also called Neu) lead to constitutive activation of the receptor. This observation led Gullick and collaborators to show that expression in cells of short transmembrane mErbB2 proteins lacking any other interacting domains reduced cell growth both *in vitro* and *in vivo* (Lofts et al., 1993). Thus blocking transmembrane domain-dependent mErbB2 dimerization may represent an avenue for the design of a novel type of drugs with anti-cancer properties. Indeed, after successful inhibition of brain tumor growth when disrupting the non-tyrosine kinase receptor Neuropilin-1 oligomerization by administration of a synthetic peptide antagonizing its transmembrane domain (Nasarre et al., 2010) we decided to explore how a similar strategy would apply for the inhibition of mErbB2 and how this may efficiently impact the metastatic process that remains nowadays the major challenge in breast cancer. We conducted this proof of concept experiment in an animal model of genetically induced breast tumors in which primary tumors are driven by the expression of a transforming mutant mErbB2 (NeuNT, exhibiting a mutation in its transmembrane domain leading to the constitutive activation of the receptor) oncogene under the MMTV mammary specific promoter (MMTV-NeuNT). Besides allowing the analysis in fully immune-competent mice, this model also gives rise to lung metastasis and has been clearly shown to be highly predictive of drug efficacy in human (Roberts et al., 2012). Here, we report that long term tri-weekly intraperitoneal administration of low doses of a synthetic Membrane Targeting Peptide (MTP) mimicking the transmembrane domain of NeuNT (MTP-NeuNT) triggers sustained inhibition of the Akt pathway thereby impeding tumor cell growth and metastasis. Hence, this proof of concept experiment demonstrates that drugs targeting the transmembrane domain of tyrosine kinase receptors offer a credible alternative to classical approaches focused on extra or intracellular domains.

Results and Discussion

Molecular modeling of NeuNT transmembrane domain

The substitution of a valine for a glutamic acid in the TMD of the mErbB2 receptor leads to the constitutive activation of the receptor in a dimeric state and carcinogenesis (Weiner et al., 1989). We first performed molecular modeling of TMD dimerizing interface to better predict the interaction of a TMD-NeuNT mimicking peptide (MTP-NeuNT) with the native TMD. Figure 1A shows the MTP-NeuNT in the context of the nearly full length dimeric NeuNT receptor. This model is based on the recently published molecular dynamics study of EGFR dimers (Arkhipov et al., 2013). Our approach illustrates how the transmembrane domain kindly accommodates to the interface of the active receptor as previously suggested (Bagossi et al., 2005). Our model supports the idea of an active role of the TMD to control the proper dimeric and conformational organization of the receptor as recently described for EGFR (Endres et al., 2013). The crossing angle of the transmembrane helices at -45 degrees involves small residues in the core of the dimer. Such conformation was shown to be among the most stable in previously published ErbB2 TMD models (Bocharov et al., 2010). Thus, in the case of NeuNT receptor, MTP-NeuNT peptide would inhibit signaling by interfering with homodimerization of the TMD of the NeuNT receptor through direct competition for binding to the dimerization interface. When locked within the dimerization interface, MTP-NeuNT peptide would also prevent further heterodimerization by reducing its availability for any other interactions.

NeuNT transmembrane domain sequence exhibits highly specific interactions

To address the specificity of the MTP-NeuNT peptide mimicking the native transmembrane domain of the NeuNT receptor we performed a two hybrid-like screening using the BACTH system (Karimova et al., 2001). We conducted a systematic approach by co-expressing

constructions encoding several TMD containing GxxxG motifs for measurement of interactions with NeuNT TMD (Figure 1B). This assay confirmed the high propensity of the MTP-NeuNT peptide to dimerize with NeuNT TMD when compared with positive control GPA-GPA interaction or negative GPAm-GPAm interaction (mutated non-dimerizing version of Glycophorin-A TMD in which glycine 83 is replaced by an isoleucine). This MTP-NeuNT/NeuNT interaction is specific because the MTP-NeuNT TMD sequence showed no hetero-dimerization with various GxxxG-containing sequences including glycophorin A (Figure 1B), Neuropilin-1, Vascular endothelial growth factor receptor 1, Vascular endothelial growth factor receptor 2, Vascular endothelial growth factor receptor 3 or Integrin Beta 1 (see supporting information figure 1). The MTP-NeuNT sequence showed strong hetero-dimerization capacity with the mouse ErbB2 sequence (mErbB2) or the human ErbB2 sequences (hErbB2). Moreover, the MTP-NeuNT sequence significantly interacted with the murine orthologous of ErbB1 (mErbB1), ErbB3 (mErB3) and ErbB4 (mErbB4) but not with the human orthologous of these receptors (figure 1B and supporting information figure 1). Thus, this part of the study confirmed stable homo- and hetero dimer formation of the ErbB2 TMD (Bennasroune et al., 2004; Cymer and Schneider, 2010) and allowed us to define the dimerizing and antagonizing capability of the MTP-NeuNT peptide while demonstrating the specificity of interactions.

MTP-NeuNT exhibits a long lasting inhibitory effect

There was no cellular model allowing the study of NeuNT signaling in a native context. To circumvent this issue we developed a cell line derived from tumors collected in MMTV-NeuNT mice (NT193 cell line). This cell line exhibits epithelial and metastatic properties and constitutively expresses the mutated version of ErbB2. We performed a dose-response analysis to characterize the inhibition of cell proliferation that was obtained with 10^{-8}M of the peptide reaching a maximal effect at 10^{-6}M (-21% at 10^{-6}M versus vehicle, $p < 0.001$, Mann

Whitney test) (Figure 1C). Moreover, we found that treating the cells with 10^{-6}M had a prolonged and intensified effect as seen by a significant 2-3 fold amplification of the inhibitory effect when measuring cell proliferation at 48h and 72h post treatment (-68% at 48h and -41% at 72h compared to control, $p < 0.001$ Mann Whitney test). This long lasting effect is dose dependent because 10^{-7}M of the MTP-NeuNT peptide only showed mild reduction over time (-17% at 24h, $p < 0.0001$ versus -4% at 48h, $p = 0.24$, and -12% at 72h, $p < 0.0004$, compared to control, Mann Whitney test) (Figure 1C). This persistent effect is consistent with our previous confocal microscopy observations demonstrating that similar TMD peptides enter the cells within a time frame of 30-50 minutes and that they can be imaged at the membrane up to 72 hours post incubation before endocytosis and degradation (Nasarre et al., 2010; Popot et al., 2011). Hence, we verified that MTP-NeuNT inhibited proliferation of another murine breast cancer cell line (4T1 cells) and ErbB2 expressing human breast cancer lines (MCF7, SKBR3) but had no effect on human breast cancer MDA-MB-231 cells lacking expression of ErbB2 at the protein level (supporting information figure 2).

MTP-NeuNT inhibits ErbB2 phosphorylation and Akt phosphorylation

Since we observed a negative impact of MTP-NeuNT on tumor cell expansion we investigated how this peptide affects ErbB2 signaling. First, we determined the phosphorylation of ErbB2 in the presence of MTP-NeuNT using a phospho-specific ELISA assay. We observed a significant 45% reduction of ErbB2 phosphorylation (normalized to total ErbB2) compared to control conditions (LDS without peptide) in NT193 cells ($p = 0.0078$, Mann Whitney test, Figure 1E). Moreover, we also found a 58% decrease of Akt phosphorylation normalized to total Akt ($p < 0.0001$, Chi-square test) in NT193 cells exposed to 10^{-6}M of MTP-NeuNT for 1 hour (Figure 1F). Strikingly, we found sustained inhibition of Akt phosphorylation up to 72h consistent with long term inhibition of cell proliferation (see

supporting information figure 3). Similarly, Erk1/2 phosphorylation (the second most important signaling pathway triggered by NeuNT/ErbB2) was reduced in the presence of the peptide (see supporting information figure 3). Hence, MTP-NeuNT is able to inhibit the pro-proliferative and survival signaling cascades triggered by the NeuNT/ErbB2 receptor. From a therapeutic point of view, the inhibition of the Akt pathway highlights an interesting anti-tumoral potential of the peptide since the Akt pathway is hyperactive in more than 70% of breast cancers (Grunt and Mariani, 2013) and is considered as one of the major source of tumor cell survival and metastasis (Kim and Chung, 2002).

MTP-NeuNT improves overall survival of MMTV-NeuNT mice

MMTV-NeuNT transgenic mice develop stochastic mammary adenocarcinoma approximately from 12 weeks of age (Muller et al., 1988). To determine how MTP-NeuNT would affect breast tumor growth we administrated intra-peritoneal bolus of 15 µg/kg of the peptide (a concentration equivalent to 10^{-6}M) 3 times a week starting when the first palpable tumor reached a volume of 200 mm^3. The experiment was finished when tumors reached the ethical limit-point as defined in the method section. Tumor occurrence was identical in both groups (100%) within a similar time frame lasting from birth to measurable 200 mm^3 tumors (214 days to reach 200 mm^3 in control versus 218 days in MTP-NeuNT group, $p = 0.95$, Mann Whitney test) before administration of treatments. A waterfall plot of best response (determined between day 21 and day 28 of treatment according to the RECIST criteria) revealed that 100% of the treated animals responded to the treatment with 33% of SD (Stable Disease, < -30% decrease of target lesion) and 67% with PR (Partial Response > 30% decrease of target lesion) (Figure 2A). This high response rate translated into a marked survival benefit (Figure 2B) with a median survival increase up to 122% compared with untreated animals (73.5 days in control animals versus 90 days in MTP-NeuNT treated animal, $p = 0.0182$, Log-rank test). We also found that the delay for the appearance of second

tumors (defined as the second palpated tumor) was doubled in MTP-NeuNT treated mice as compared to control mice (4.6 days in control group versus 10.4 days in MTP-NeuNT treated animals, p = 0.0066, Mann Withney test) thereby demonstrating an overall impact on disease progression. In comparison to our results for MTP-NeuNT that already reduced primary tumor onset and growth, a genetically delivered TGFβ antagonist had no effect on tumor latency in MMTV-NeuNT mice (Yang et al., 2002). Thus, antagonizing the TMD of NeuNT appears as a potent therapeutic strategy.

Anti-metastatic activity of MTP-NeuNT

The occurrence of lung metastasis is a critical step in breast cancer progression and linked to disease-associated death (Chambers et al., 2002). Thus, we decided to monitor whether the improved survival of mice treated with MTP-NeuNT could reflect reduced lung metastatic colonization. To this end we analyzed the serial µCT images of the whole animals collected at different time points of the protocol. Figure 2C is presenting representative examples of the metastases detected in the control or the MTP-NeuNT treated group (3D surface rendering or sagittal and axial views). A waterfall plot of best response (determined between week 7 and week 8 of treatment according to the RECIST criteria) revealed that 100% of the treated animals responded to the treatment with 20% of SD (Stable Disease, < -30% decrease of target lesion) and 80% with PR (Partial Response > 30% decrease of target lesion) including two individuals above 90% of decrease in metastasis volume (Figure 2D). Quantitative measurements of the total number of metastasis in randomly selected 5/10 control mice and 5/10 MTP-NeuNT mice revealed a 2.4 fold decrease (n=26 detectable metastases in control versus n=11 metastases in MTP-NeuNT group) in treated animals after eight weeks treatment (Figure 2E). Determination of metastases volumes also revealed a 4.5 fold decrease when comparing cumulated volumes of metastases in mice of MTP-NeuNT group with those of the control group (cumulated volume CV = 78.3 mm^3 in control versus CV = 17.5 mm^3 in MTP-

NeuNT group) (Figure 2F). Hence, µCT analysis revealed a significant anti-metastatic effect of MTP-NeuNT characterized by both a reduction of the number and size of the lesions. Because micro-metastases (< 1 mm in diameter) are difficult to analyze by µCT we decided to perform a detailed histological examination of the lungs to further analyze the anti-metastatic effect of MTP-NeuNT.

MTP-NeuNT inhibits metastasis proliferation and prevents parenchymal invasion

To further explore the anti-metastatic effect of MTP-NeuNT we performed a systematic histological examination of the lungs. This allowed us to confirm an overall 1.8 fold reduction of the mean number of lung metastasis (Figure 3). Interestingly, a detailed histological analysis allowed us to distinguish between intravascular and parenchymal metastases (Siegel et al., 2003) to reveal a 2.6 fold decrease of the number of parenchymal metastasis in the MTP-NeuNT treated group compared to the control group. This result suggests that the peptide is able to reduce parenchymal invasion, presumably because impeding extravasation of metastasizing cells forming emboli in blood vessels. We also measured the proliferative activity of lung metastases using Ki67 staining. Consistently with our *in vitro* work we found a general 2.7 fold decrease of tumor cell proliferation (Figure 3B). Moreover, consistent with the observed reduction of the size of metastases we found a 1.9 fold increase of TUNEL positive cells in the metastases of treated animals illustrating that the peptide impaired tumor cell survival (Figure 3C). Hence, we found a general 4.9 fold decrease of Akt phosphorylation in situ (Figure 3D). Altogether these data demonstrate that the therapeutic peptide is able to slow down proliferation and extravasation of metastatic cells and survival as a consequence of Akt pathway inhibition.

Conclusive remarks

We believe that the transmembrane domain of tyrosine kinase receptors offer a novel dimension for drug design because this underestimated domain is crucial for the control of receptor activation (Arkhipov et al., 2013; Endres et al., 2013). Our study demonstrates that a peptide mimicking the TMD of the mutant mErbB2 (NeuNT) is able to specifically antagonize NeuNT, mouse and human ErbB2 thereby inhibiting phosphorylation of the receptor and downstream signaling. This long lasting inhibitory property translated into improved survival and strong reduction of metastasis growth of mice presenting genetically induced mammary tumors. Strikingly, micro-molar dosage was sufficient to trigger significant therapeutic benefit with no histological sign of toxicity (supporting information figure 4). This unique strategy may apply to other tyrosine kinase receptors therefore opening a novel avenue for drug design.

Experimental Procedures

Cell culture

All cell media were supplemented with 10% fetal calf serum (FCS) (GIBCO), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma), and cultures were performed at 37ºC, 5% CO2. Cultures were grown up to 70-80% confluence and were routinely split in 10 cm culture dishes. Human epithelial breast adenocarcinoma derived from pleural effusion MDA-MB-231 (ErbB2 negative), MCF7 (ErbB2 positive), SKBR3 (overexpressing ErbB2) cells were grown in Dulbecco's modified Eagle medium (DMEM, GIBCO). Murine Balb/c 4T1 (ErbB2 positive) cells derived from a spontaneous tumor were grown in Roswell Park Memorial Institute medium 1640 (RPMI, GIBCO). MDA-MB-231 cells were purchased from ECACC (92020424), MCF7 and 4T1 cells were obtained from our institute collection, MDA-MB-231 were obtained from Dr Jacky Goetz. NT193 cells were derived from MMTV-NeuNT primary

tumors. In brief, breast tumor from MMTV-NeuNT mice was removed upon sacrifice, minced finely using scissors and subjected to enzymatic digestion using 0.25% trypsine in Versene solution. The cellular suspension was passed through 40 μm nylon filter (BD Falcon) and cultivated as describe above. The morphology of the cells was analyzed continuously. Fibroblast-like cells were removed by selective trypsinization. Briefly, cells were washed with PBS and mesenchymal-like cells were removed after a short (2 to 5 min) treatment with a solution of trypsin at low concentration (0.05% w/v trypsin, 0.02% w/v EDTA in PBS). Detached cells were removed, still adherent (epithelial) cells were then washed with PBS once and the medium was re-freshed.

Peptides

Peptides have been synthesized by the Peptide Specialty Laboratories GmbH by automatic peptide synthesis (Fmoc chemistry). The peptide corresponding to the TM sequence of NeuNT : TFIIATVEGVLLFLILVVVVGILIKRR (in one-letter code, amino acid T^{654} to R^{680} according to Swissprot entry P06494) is referred as MTP-NeuNT. Peptides purity estimated by RP-HPLC was more than 90% according to manufacturer indication.

Modeling of the NeuNT receptor dimer and MTP-NeuNT

The nearly full length of the monomeric and the dimeric ErbB2 receptors were modeled by homology using MODELLER 9.11. The multi templates strategy was employed using as templates the recent model of EGFR obtained by molecular dynamics [24] and the X-rays and NMR structures of the domains available for the murine ErbB2 receptor. These domains are the extracellular region (PDB.ID 1N8Y), the kinase domain (PDB.ID 3PP0) and the right-handed dimer model of the TM domain [39]. The 3D structure of the missing part of the intracellular domain of the murine ErbB2 was modeled using the prediction server I-

TASSER. The homology models of the monomeric and the dimeric ErbB2 receptors were energetically minimized before their insertion in a lipidic bilayer.

Estimation of MTP-NeuNT dimerization (BACTH Method)

A bacterial two-hybrid system based on the recombination of adenylate cyclase CyaA from Bordetella pertussis was used to measure both homo- and heterodimerization propensities of transmembrane domains of interest. This system relies on reconstitution of the catalytic domain of CyaA which can be separated into two complementary fragments [29]. When each fragment is fused to a protein of interest, a functional adenylate cyclase can be reassembled upon interaction of the two proteins, which is followed by the production of cyclic AMP in an *E. coli* strain lacking its own adenylate cyclase. The synthesis of cyclic AMP is proportional to the association propensity of the two fragments, and can be measured in a number of ways [40]. This system has been used widely and is readily amenable for study of membrane proteins interactions. We have adapted the BACTH system for the study of homotypic and heterotypic interactions of TMD (Sawma et al, under revision). Very briefly, we have modified the BACTH pKTN25 and pUT18 plasmids so that they encode for hybrid proteins containing a signal sequence followed by the different TMDs of interest and the T25 and T18 fragments of adenylate cyclase. Double transformation in BTH101 (cya-) *E. coli* cells, cell growth and induction, and beta-Galactosidase assay in 96-well arrays on a TECAN machine were performed as described [40]. Peptide sequences used in this study are summarized in supplementary table 1.

Immunocytochemistry

Cells were grown on sterile glass cover slips for one day before immunofluorescence staining. The cells were fixed with freshly made fixative 4% formaldehyde (FA) for 10 minutes. The samples were gently rinsed with PBS (1 wash for 10 minutes) before adding the blocking

solution (FCS 5%) for a minimum of 30 minutes. Cells were permeabilized using 1x PBS with 0.1% Tween20 for 5 minutes. Primary HER2/erbB2 (Cell signaling #2242, diluted 1/50 from stock solution) antibody diluted in 5% fetal calf serum (FBS)-PBS was added to the cell over night at room temperature. After thorough wash (3 washes of 5 minutes) secondary antibody (donkey anti-rabbit, Jackson Immunoresearch 711-165-152 diluted 1/1000 from stock) was added at room temperature for 90 minutes. After washing in PBS, the cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole, 1/30000 in water) for 10 minutes. Glass coverslips were finally mounted on microscopy glass slides using a polymerization medium (FluorSave reagent, Calbiochem-Merck cat#345789).

Histology

Giemsa staining

Lung metastasis production was analyzed at the histological level using Giemsa staining. After mice sacrifice, lung (left lobe) were removed and fixed overnight in 4% FA. Following extensive wash (at least 3 washes of 15 minutes) organs were dehydrated in 100% ethanol for a day and then embedded in paraffin. Samples were stored at RT. The paraffin embedded tissue blocks sections (7µm thickness) were de-waxed and rehydrated through 100% Toluene (2 washes of 15 minutes) then 100% alcohol (2 washes of 15 minutes), 95% alcohol, 90% alcohol, 70% alcohol and water (1 wash 10 minutes respectively) and then stained with freshly made up Giemsa stain (RAL#320310-0125, diluted 1/50 from stock solution) for 2h at 37ºC. After extensive washing, differentiation is achieved with 0.5% aqueous acetic solution for 30 seconds. Sections are then rapidly dehydrated in 70% alcohol, 90% alcohol, 95% alcohol, 100% alcohol with rapid dips in each bath before final wash in toluene for 15 minutes. Slides were mounted in Eukitt (Sigma) for long term conservation and microscopic observation.

Hematoxylin-eosin

The paraffin embedded kidney and liver sections were dewaxed and rehydrated as described above. Sections were stained with hematoxylin (Surgipath # 01562E) for 5 minutes and washed with running tap water. Differentiation was accomplished in acid alcohol solution for 7 seconds followed by thorough wash (running tap water for at least 10 minutes). Sections were incubated in eosin (Harris, RAL# 31273-7) for 10 seconds, rinsed again and dehydrated as described above before mounting in Eukitt (Sigma #03989).

Cell proliferation

In vitro cell proliferation was monitored using MTT (3-(4,5 -Dimethylthiazol-2 -yl)-2,5-di phenyl tetrazolium bromide) proliferation assay according to manufacturer's instruction (Sigma, M2128, USA). Optical density was determined at 570 nm using an ELISA plate reader spectrophotometer (EL800, Bio-Tek Instruments). NT193 cells were seeded at a density of 10 000 cells per well in a 96 well plate, the cells were then incubated with increasing peptide concentration (ranging from 10^{-9}M to 10^{-6}M) or corresponding vehicle increasing concentration (LDS, ranging from 0.72µM to 720µM). This assay allowed us to determine acute toxicity of the treatments when performed after 4h treatment (10^{-7}M) while cell proliferation was evaluated after 24h, 48h and/or 72h. After desired incubation period the culture media were removed from the well and 100 µl of MTT dye freshly diluted (to 1/50 in GBSS) from stock solution (5mg/ml) was added to each well for 4h. After this incubation period, isopropanol (100µl) was added to the MTT solution in each well before reading the optical density at 570 nm.

In vivo cell proliferation was determined by quantification of Ki67 positive cells. Sections (7µm thickness) were dewaxed as described above. Tissue was boiled with the antigen retrieval sodium citrate buffer (pH = 6) for 20 min (Sigma #CO759) in order to restore

antigenic reactivity. After incubation with blocking solution (PBS Triton-100X +5% NGS) for 20 minutes, the avidin/biotin blocking solutions were added on the tissue for 15 minutes respectively (Blocking kit from VECTOR laboratories SP-2001). The primary anti-Ki67 antibody (ThermoScientific, RM-9106) was added to the specimen in blocking solution overnight at a dilution of 1/200. An extensive washing was then completed (5x1min in PBS) before incubation with the secondary antibody (Goat anti-rabbit, Vector Laboratories, #PI-1000) at a dilution of 1/200 in PBS for 1 hour at room temperature. The tissue was rinsed with PBS (2x2 minutes) and then the endogenous peroxidases were blocked with 1% H_2O_2 solution (Sigma, #H1009). This later solution was washed out and amplification of the signal was achieved using the ABC peroxidase solution (Elit Vectastain kit, PK-6100). DAB was finally added (kit from Vector laboratories). All specimens were counterstained with Hematoxylin to simplify tissue architecture evaluation (30 seconds). Lastly sections were dehydrated as described previously and mounted in Eukitt (Sigma, #03989).

Determination of apoptosis

Sections were dewaxed and rehydrated as described above. Apoptosis was determined using the 'ApopTag Plus Peroxidase *In situ* Hybridization and Detection Kit' (Chemicon, S7101, CA, USA) according to the manufacturer's instructions. Number of positive cells was counted on a total of 8 sections of the control group and 8 sections of the MTP-NeuNT treated group. Data were expressed as the number of positive (apoptotic) cells per area (µm2).

Image acquisition

Cell fluorescence images and high magnification images were acquired with the fluorescence Zeiss Imager Z2 equipped with HXP 120W lamp and ApoTome system. Lung metastasis and low magnification images were acquired using a macroscope Zeiss AXIOZoom.V16. Images

were analyzed using AxioVision 4.7.2 (Zeiss), Zen (Zeiss) and Image J (Wayne Rasband, NIH) software.

Phosphorylation of ErbB2 receptor on NT193 cells

Phospho-Neu (Y1221/Y1222) cell-Based colorimetric ELISA Kit (*Immunoway* Biotechnology Company, Newark, DE, USA) was used to monitor levels of phosphorylated mErbB2 and related total mErbB2 receptor. NT193 cells were seeded into the wells of the 96 well plate at densities of 30 000 cells in 200µl cell culture media and serum starved overnight. The cells were then treated with either vehicle (LDS 720µM) or MTP-NeuNT peptide for 1h at 10^{-6}M. mErbB2 phosphorylation was assessed according to the manufacturer's instructions.

Western blot

NT193 cells were seeded at a density of 600 000 cells per well in a 6 well plate and serum starved overnight. Before protein extraction, the cells were treated either with 10^{-6}M of MTP-NeuNT or Vehicle (LDS, 720µM). After a rapid PBS wash, the protein sample preparation was completed with Laemmli buffer (100 mg/ml SDS, 250 µl/ml TRIS 1M pH=6.8, 1 mg/ml Bromphenol Blue, 77 mg/ml Dithiothreitol, 500 µl/ml Glycerol , 500 µl/ml H_2O) supplemented with protease inhibitor (Roche tablets, #11836145001) and 5 mM of Na orthovanadate. Proteins were resolved in a 8% SDS/PAGE gel and transferred onto a nitrocellulose membrane (Whatman). The blot was soaked in blocking solution (TBS/1%tween/5%milk) for 1h at RT. First antibodies (Rabbit anti-phospho-akt, Rabbit anti-akt, Cell Signaling #4060 and #9272 respectively) and mouse anti-actine (Chemicon #MAB1501R) were incubated overnight at 4°C. Succeeding washing (3 times 5 minutes, TBS/tween 1%), secondary antibodies (anti-rabbit-HRP, GE Healthcare, #NA934V and anti-mouse-HRP, GE Healthcare, #NXA931) were incubated 1h a RT in TBS/1%tween /5%milk. The revelation step was performed using streptavidin-biotinylated Horseradish Peroxidase

complex (Amersham #RPN1051) according to the manufacturer instructions. Images of the immune-blots were acquired and analyzed thanks to the GENE GNOME apparatus (Syngene Bio Imaging, UK).

Animal handling and *in vivo* ethical statement

Experiments were performed according to the Guide for Care and Use of Laboratory Animals (E67-6-482-21) and the European Directive with approval of the regional ethical committee (Reference AL/55/62/02/13). Mice received food and water ad libitum. Animals were sacrificed using CO_2. All necessary precautions were taken to minimize pain or discomfort of the animals. General health status was monitored 3 times a week by independent observers. Sacrifice of the animal was effectuated when reaching limit ethical endpoints.

Production of the transgenic MMTV-NeuNT mice

Transgenic FVB mice expressing the mutant activated form of murine ErbB2 (NeuNT) under control of the mouse mammary tumor virus (MMTV) promoter (Muller et al., 1988), were kindly obtained from Dr Gerhard Christofori (University of Basel). Mice were maintained under strict inbreeding conditions crossing FVB females with MMTV-NeuNT +/- males; the presence of the NeuNT transgene was checked by PCR on tail DNA two weeks after birth in females (forward primer NeuNT_F: 5'-GGAAGTACCCGGATGAGGAGGGCATATG-3', reverse primer NeuNT_R: 5'-CCGGGCAGCCAGGTCCCTGTGTACAAGCCG-3'). We generated a cohort of 20 mice receiving either 15µg/kg of MTP-NeuNT or peptide vehicle (LDS, 720µM) three times a week. Treatments were administrated when first palpated tumor reached 200 mm^3 volume as determined using electronic caliper based measurements of the tumor diameter. Upon sacrifice of the mice, lungs, kidney and liver were removed and collected for histological examination.

In vivo imaging and surface rendering of µCT

In vivo µCT, equipped with dedicated anesthesia chamber, was performed on a weekly basis allowing pulmonary metastasis monitoring. The cone beam micro-CT system has been constructed at the IPHC (Institut Pluridisciplinaire Hubert Curien), Strasbourg, France. It consists in an X-ray source, an x-ray detector and 3D translation and rotation stages. The x-ray source is a commercially sealed tube (L9181-02, Hamamatsu) with a tungsten anode, a 200 µm beryllium and a 1 mm Aluminum exit windows. The source has an 8 µm focal spot and a maximum output power of 8 W. With a selected output power less than 4 W, the size of the focal spot is 5 µm. The x-ray source operates in continuous mode with a 39º maximum beam angle. The x-ray detector is a commercially available flat panel sensor (C7942CA-22, Hamamatsu) composed of a CsI scintillator plate coupled to 1 mm Aluminum filter and a two-dimensional photodiode array (CMOS) leading to an active area of 120 x 120 mm2 and 2400 x 2400 active pixel elements. The pixel size is 50 x 50 μm^2. Acquisition parameters were 40 kVp, 250 lA and 235 ms exposures per projection in binning 2 x 2 with a projection pixel size of 100 x 100 cm^2. A full-image data set covered 360° in 0.47° steps for a total of 768 projections. The continuous rotation of the system during the acquisition resulted in a total acquisition time of 3 minutes. Images were reconstructed with a cone-beam reconstruction algorithm producing in real time a 3-D image with an isotropic voxel size of 0.1 mm. The delivered dose during each microCT exam was 48 mGy. Once the metastasis was detected in the lung, its volume was calculated as follows: $V = (4/3) * \pi * rx * ry * rz$, where r is the radius of the metastasis and x, y, z are the axial directions.

Statistics

Statistical analyses were performed using Mann Whitney test (for sample $n < 30$), Chi square analysis (for qualitative data), Extra sum of square F test (for the µCT number of metastasis

curves) using GraphPad software (USA). P-values are given in the figure legends, and values of $P < 0.05$ were considered to be statistically significant. Normal distribution of the values was checked using GraphPad software (USA). A minimum of three independent experiments was performed for *in vitro* assays (proliferation, toxicity, ErbB2 phosphorylation and Western blot). For *in vivo* experiment sample size calculation anticipated a therapeutic effect of 20% for a standard deviation of 14% and confidence interval of confidence 95% (Lamorte's Power calculation, University of Boston).

Acknowledgements

The authors are grateful to D. Gerhard Christofori for providing the MMTV-NeuNT model and to Florian Busso and Abdoul Ahad Ould Sidi for excellent technical support for the 3D surface rendering of lung metastastases. This work was supported by INCA to GO, ANR to DB/PH/MG and Ligue Régionale contre le Cancer to DB.

Conflict of Interest: the authors declare no conflict of interest

References

Arkhipov, A., Shan, Y., Das, R., Endres, N.F., Eastwood, M.P., Wemmer, D.E., Kuriyan, J., and Shaw, D.E. (2013). Architecture and membrane interactions of the EGF receptor. Cell *152*, 557–569.

Arteaga, C.L., Sliwkowski, M.X., Osborne, C.K., Perez, E.A., Puglisi, F., and Gianni, L. (2012). Treatment of HER2-positive breast cancer: current status and future perspectives. Nat. Rev. Clin. Oncol. *9*, 16–32.

Bagossi, P., Horvath, G., Vereb, G., Szollosi, J., and Tozser, J. (2005). Molecular Modeling of Nearly Full-Length ErbB2 Receptor. Biophys. J. *88*, 1354–1363.

Bennasroune, A., Fickova, M., Gardin, A., Dirrig-Grosch, S., Aunis, D., Crémel, G., and Hubert, P. (2004). Transmembrane peptides as inhibitors of ErbB receptor signaling. Mol. Biol. Cell *15*, 3464–3474.

Bocharov, E.V., Volynsky, P.E., Pavlov, K.V., Efremov, R.G., and Arseniev, A.S. (2010). Structure elucidation of dimeric transmembrane domains of bitopic proteins. Cell Adhes. Migr. *4*, 284–298.

Bombonati, A., and Sgroi, D.C. (2011). The molecular pathology of breast cancer progression. J. Pathol. *223*, 307–317.

Chambers, A.F., Groom, A.C., and MacDonald, I.C. (2002). Dissemination and growth of cancer cells in metastatic sites. Nat. Rev. Cancer *2*, 563–572.

Charafe-Jauffret, E., Tarpin, C., Bardou, V.-J., Bertucci, F., Ginestier, C., Braud, A.-C., Puig, B., Geneix, J., Hassoun, J., Birnbaum, D., et al. (2004). Immunophenotypic analysis of inflammatory breast cancers: identification of an "inflammatory signature."J. Pathol. *202*, 265–273.

Cymer, F., and Schneider, D. (2010). Transmembrane helix-helix interactions involved in ErbB receptor signaling. Cell Adhes. Migr. *4*, 299–312.

Eccles, S.A. (2011). The epidermal growth factor receptor/Erb-B/HER family in normal and malignant breast biology. Int. J. Dev. Biol. *55*, 685–696.

Endres, N.F., Das, R., Smith, A.W., Arkhipov, A., Kovacs, E., Huang, Y., Pelton, J.G., Shan, Y., Shaw, D.E., Wemmer, D.E., et al. (2013). Conformational coupling across the plasma membrane in activation of the EGF receptor. Cell *152*, 543–556.

Freudenberg, J.A., Wang, Q., Katsumata, M., Drebin, J., Nagatomo, I., and Greene, M.I. (2009). The role of HER2 in early breast cancer metastasis and the origins of resistance to HER2-targeted therapies. Exp. Mol. Pathol. *87*, 1–11.

Grunt, T.W., and Mariani, G.L. (2013). Novel Approaches for Molecular Targeted Therapy of Breast Cancer: Interfering with PI3K/AKT/mTOR Signaling. Curr. Cancer Drug Targets *13*, 188–204.

Karimova, G., Ullmann, A., and Ladant, D. (2001). Protein-protein interaction between Bacillus stearothermophilus tyrosyl-tRNA synthetase subdomains revealed by a bacterial two-hybrid system. J. Mol. Microbiol. Biotechnol. *3*, 73–82.

Kim, D., and Chung, J. (2002). Akt: versatile mediator of cell survival and beyond. J. Biochem. Mol. Biol. *35*, 106–115.

Lofts, F.J., Hurst, H.C., Sternberg, M.J., and Gullick, W.J. (1993). Specific short transmembrane sequences can inhibit transformation by the mutant neu growth factor receptor in vitro and in vivo. Oncogene *8*, 2813–2820.

Muller, W.J., Sinn, E., Pattengale, P.K., Wallace, R., and Leder, P. (1988). Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. Cell *54*, 105–115.

Nasarre, C., Roth, M., Jacob, L., Roth, L., Koncina, E., Thien, A., Labourdette, G., Poulet, P., Hubert, P., Crémel, G., et al. (2010). Peptide-based interference of the transmembrane domain of neuropilin-1 inhibits glioma growth in vivo. Oncogene *29*, 2381–2392.

Popot, J.-L., Althoff, T., Bagnard, D., Banères, J.-L., Bazzacco, P., Billon-Denis, E., Catoire, L.J., Champeil, P., Charvolin, D., Cocco, M.J., et al. (2011). Amphipols from A to Z. Annu. Rev. Biophys. *40*, 379–408.

Roberts, P.J., Usary, J.E., Darr, D.B., Dillon, P.M., Pfefferle, A.D., Whittle, M.C., Duncan, J.S., Johnson, S.M., Combest, A.J., Jin, J., et al. (2012). Combined PI3K/mTOR and MEK inhibition provides broad antitumor activity in faithful murine cancer models. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. *18*, 5290–5303.

Ross, J.S., and Fletcher, J.A. (1999). The HER-2/neu oncogene: prognostic factor, predictive factor and target for therapy. Semin. Cancer Biol. *9*, 125–138.

Siegel, P.M., Shu, W., Cardiff, R.D., Muller, W.J., and Massagué, J. (2003). Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. Proc. Natl. Acad. Sci. U. S. A. *100*, 8430–8435.

Weiner, D.B., Kokai, Y., Wada, T., Cohen, J.A., Williams, W.V., and Greene, M.I. (1989). Linkage of tyrosine kinase activity with transforming ability of the p185neu oncoprotein. Oncogene *4*, 1175–1183.

Yamaguchi, H., Chang, S.-S., Hsu, J.L., and Hung, M.-C. (2013). Signaling cross-talk in the resistance to HER family receptor targeted therapy. Oncogene.

Yang, Y.-A., Dukhanina, O., Tang, B., Mamura, M., Letterio, J.J., MacGregor, J., Patel, S.C., Khozin, S., Liu, Z.-Y., Green, J., et al. (2002). Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. J. Clin. Invest. *109*, 1607–1615.

Figure legends

Figure 1: Demonstration of the peptide-based inhibition of NeuNT/ErbB2 receptor

(A) Analogy-based model of the nearly full length monomeric and dimeric ErbB2 receptor illustrating the interaction between native transmembrane domains (TMD) or with a peptide mimicking the TMD. (B) Bacterial Adenylate Cyclase Two-Hybrid System demonstrating the specificity and capacity of homo- or hetero dimerization of NeuNT TMD. (C) Demonstration of the dose-dependent anti-proliferative effect of MTP-NeuNT in NT193 cells derived from MMTV-NeuNT tumor-bearing mice using a MTT assay. (D) Demonstration of the prolonged and intensified anti-proliferative effect of the peptide over time. (E) Demonstration of the inhibition of ErbB2 phosphorylation (ELISA assay) and (F) Akt phosphorylation (Western blot) in NT193 cells treated with 10^{-6}M of MTP-NeuNT.

Figure 2. Effect of MTP-NeuNT *in vivo*

(A) Demonstration of the inhibitory effect of NeuNT peptide on primary tumors volumes between day 21 and day 28 of treatment. The Waterfall graph represents the percent change in tumor volume of individual treated animals (gray bars, n=9) compared to the averaged tumor volume increased determined in the control group (dark bar, n=10). This demonstrates that 100% of the treated animals responded to the treatment with 30% stable disease and 70% partial response. (B) Kaplan Meyer survival curve demonstrating a significant increased survival of the treated animals compared to the control animals. (C) Representative example of lung metastases seen from µCT 3D surface rendering or single sagittal and axial sections of the lungs in a control animal (left panel) and in a treated animal (right panel). Lung metastasis are surrounded in red, spinal cord in dashed pink, heart in dashed yellow, orange arrow heads point ribs. (D) Waterfall graph representing the percent change in metastases volume of individual treated animals (gray bars, n=5) compared to the averaged metastases volume

increase determined in the control group (dark bar, n=5) between week 7 and 8 of treatment. This plot revealed that 100% of the treated animals responded to the treatment. (E) Regression curves indicating that MTP-NeuNT treatment decreases the number of lung metastasis over time and (D) the cumulated volume of lung metastases.

Figure 3. Histological analysis of lung metastasis

(A) Representative microphotographs illustrating extravasating metastasis and pure intravascular metastasis in the control and treated group respectively. Systematic quantification of the number of metastasis revealing a 2 fold reduction in MTP-NeuNT treated animals. (B) Demonstration of the anti-proliferative activity of the peptide by systematic counting of Ki67 positive cells. (C) Demonstration of the pro-apoptotic effect of the peptide using the TUNEL method. (D) Demonstration of the inhibition of the Akt pathway by systematic counting of phospho-Akt positive cells in metastasis. (B-D) The left part is showing representative images of Ki67, TUNEL or phospho-Akt positive cells (immunocytochemistry, scale bars: 50µm). The right parts are presenting the corresponding quantitation, P-values are determined by Mann Whitney test, * = $P < 0.05$; ** = $P < 0.001$; *** = $P < 0.0001$.

.

Figure 1. Demonstration of the peptide-based inhibition of NeuNT/ErbB2 receptor

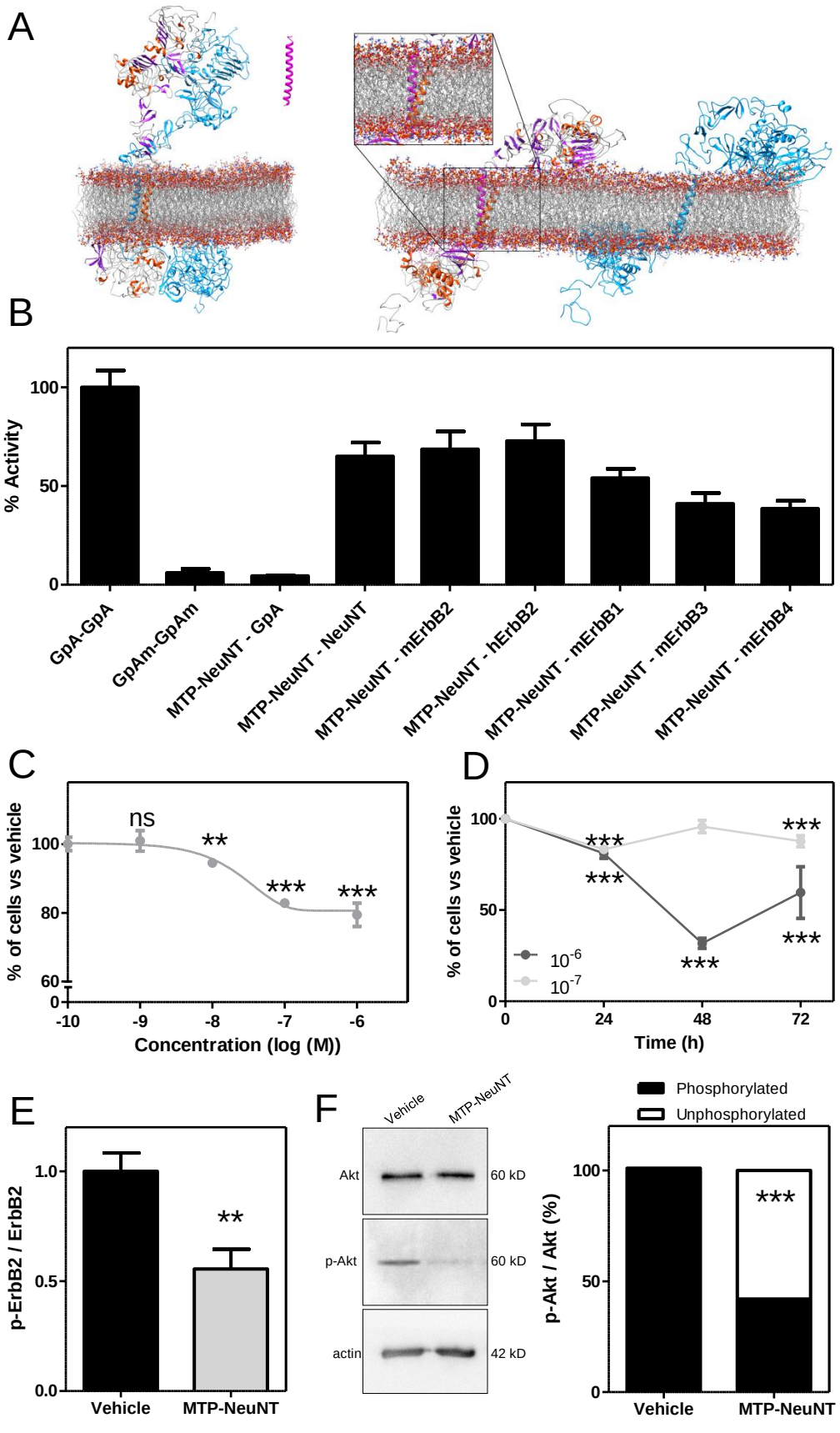


Figure 2. Effect of MTP-NeuNT *in vivo*

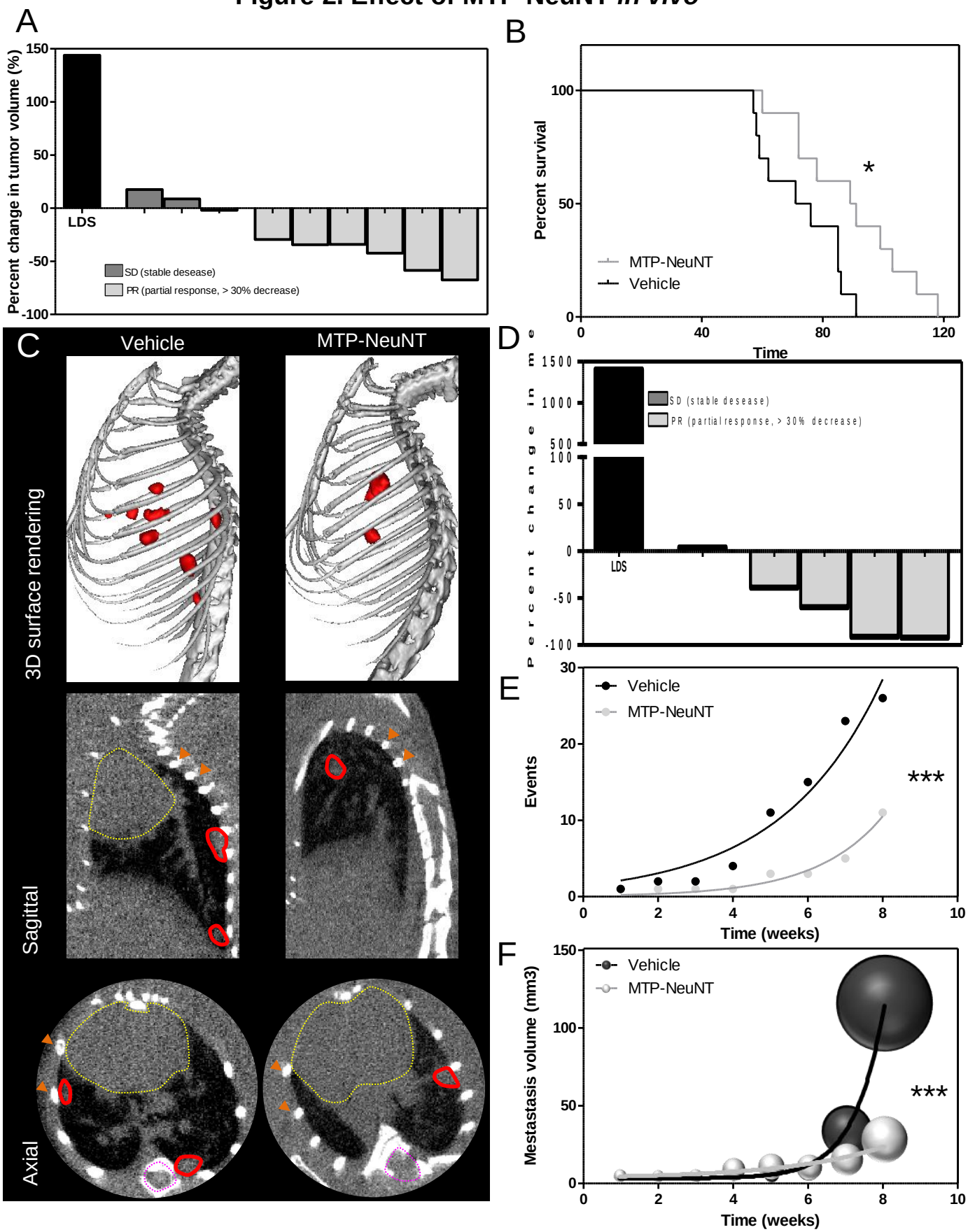
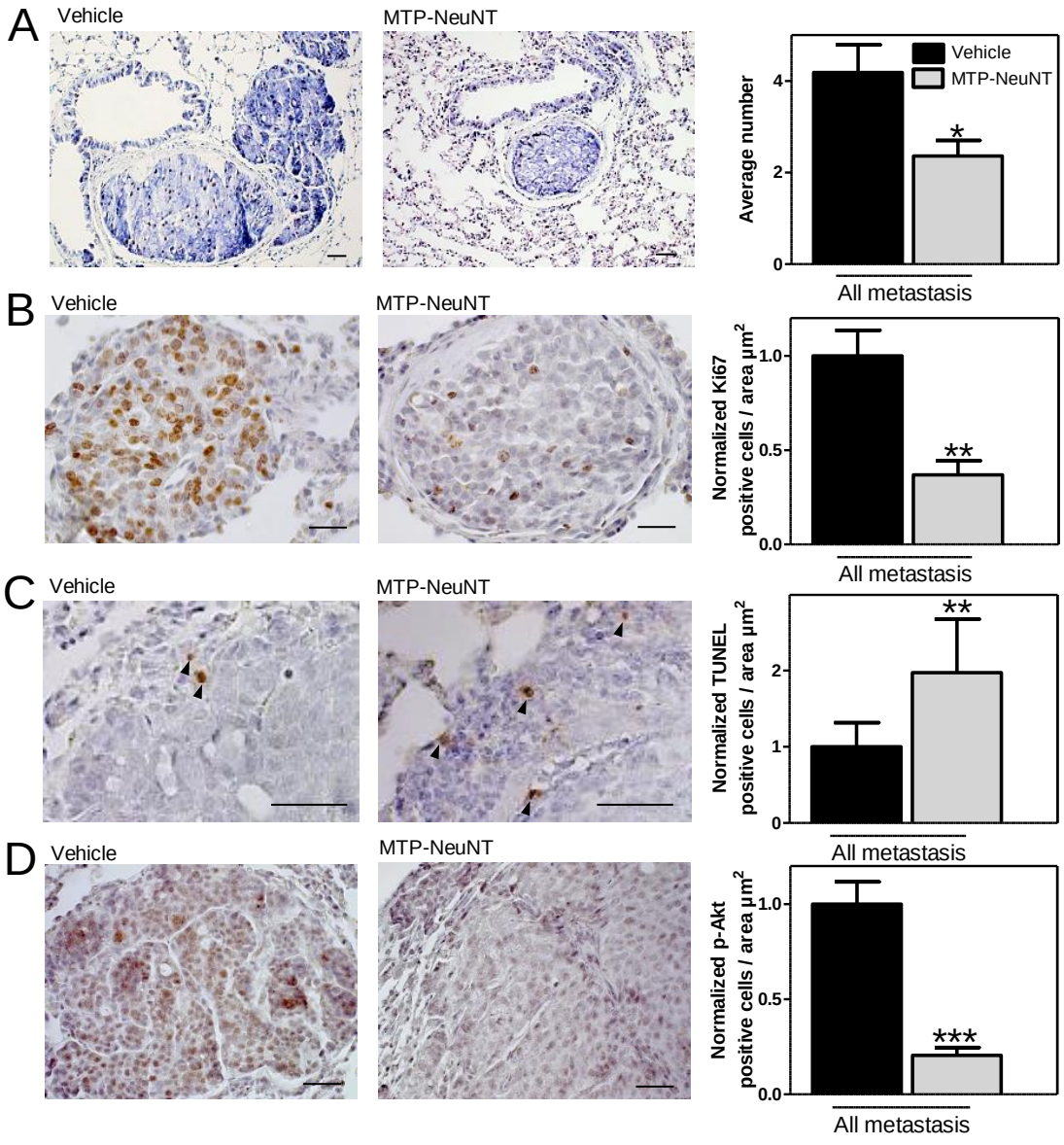


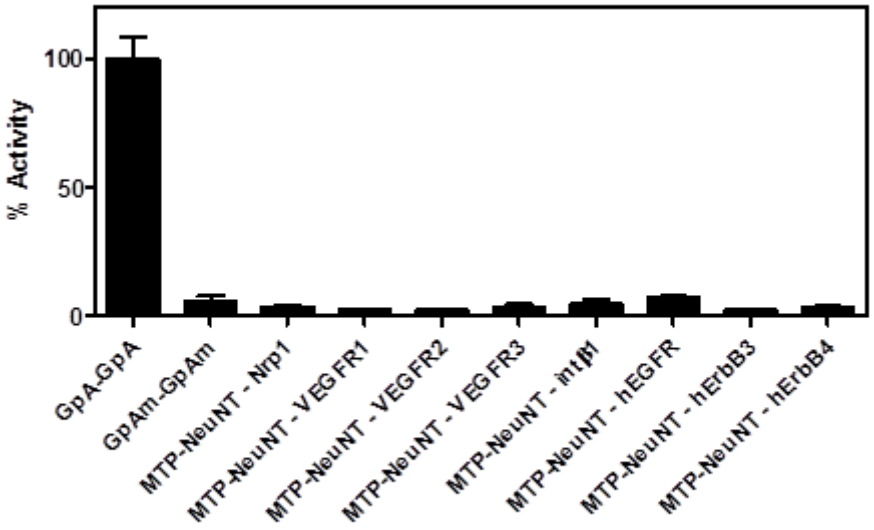
Figure 3. Histological analysis of lung metastasis



Supplemental figures – Manuscript by Arpel et al., Cell Reports

Supplemental figure 1 :

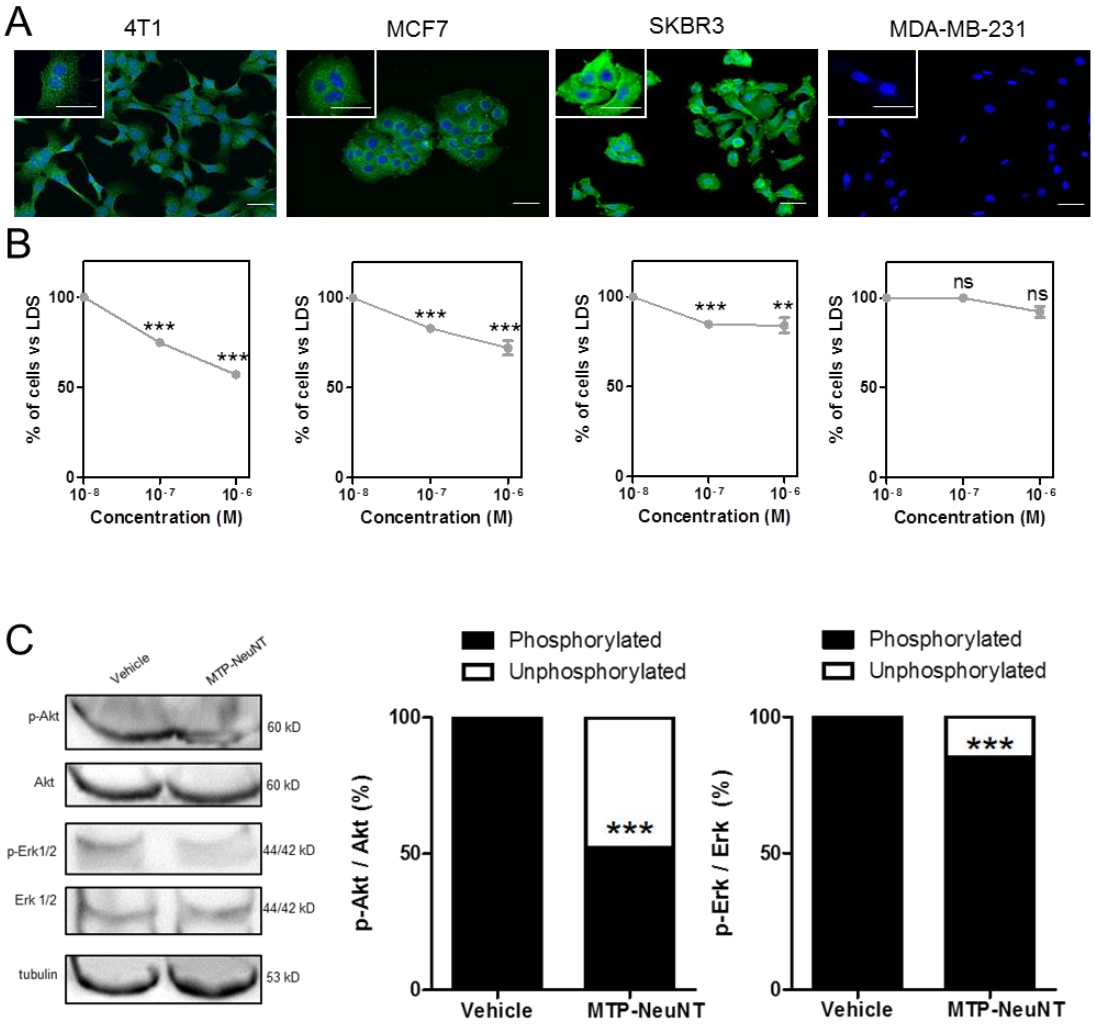
This figure is supporting figure 1 to provide reviewers with typical examples of negative interactions between the transmembrane domain of NeuNT and various transmembrane domains containing potential interacting sequences.



Determination of the specificity of the transmembrane peptides interaction. BACTH system (Bacterial Adenylate Cyclase Two-Hybrid system). Bacterias were transformed with plasmids expressing the transmembrane domain of indicated receptors. Dimerization was quantified according to β-galactosidase enzyme activity (normalized to Glycophorin A taken as 100%) as describe in Material and Methods. Nrp1= Neuropilin-1; Intb1= Integrin b-1; VEGFR1= Vascular Endothelial Growth factor-1; VEGFR2= Vascular Endothelial Growth factor-2; VEGFR3= Vascular Endothelial Growth factor-3; hEGFR= human Epidermal growth factor; hERB3= human Receptor tyrosine-protein kinase 3; hErbB4 = human Receptor tyrosine-protein kinase 4. Data are presented as mean ± SEM of three independent experiments performed in triplicates.

Supplemental figure 2:

This figure is supporting information included in figure 2 of the manuscript. Here, the long lasting inhibition of Akt and Erk1/2 pathways following addition of the therapeutic pepide is shown. Hence, additional results demonstrate that that this peptide is efficient in other ErbB2/Her2 expressing cell line but not in cells lacking expression of the targeted receptor.

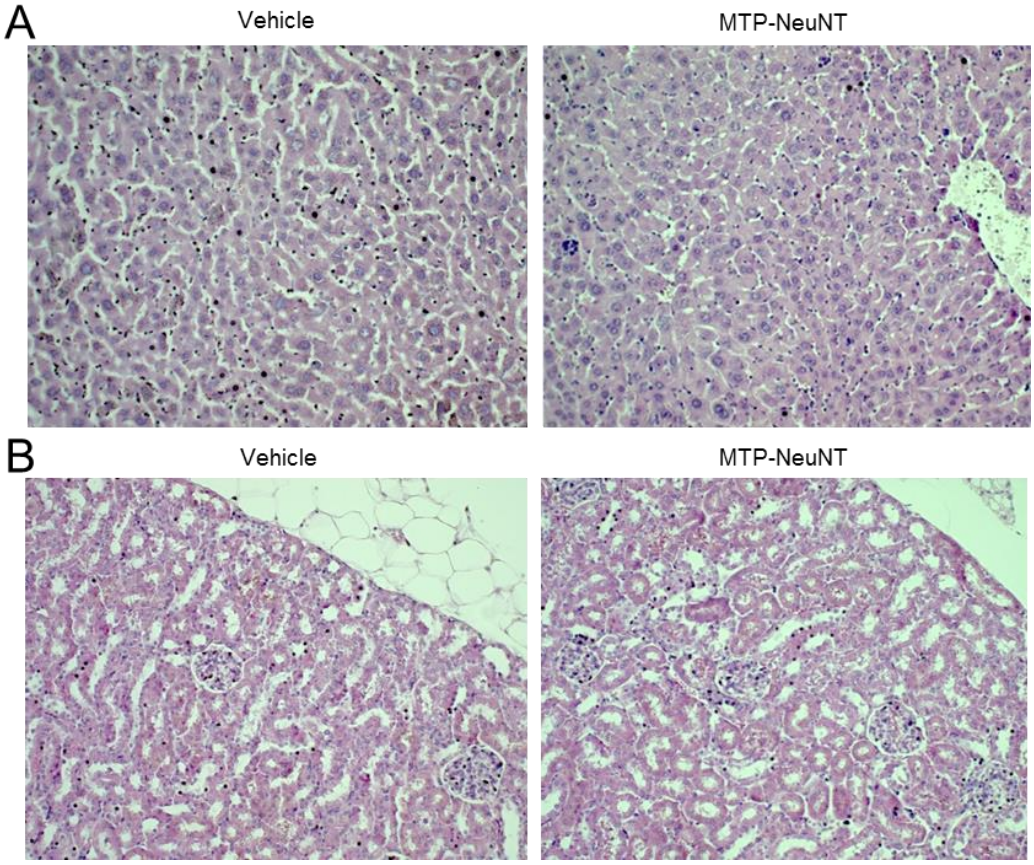


MTP-NeuNT inhibits proliferation of ErbB2-expressing breast tumor cells. (A) Immunofluorescence of ErbB2 receptor (green) and nuclei (blue) of the murine cell line 4T1, and on human cell lines MCF7, SKBR3, MDA-MB-231 from left to right respectively, validating expression or lack of ErbB2 expression at the protein level. Insets within images represent higher magnifications of different fields. Scale bars: 50 µm. (B) Proliferation assay (MTT) respectively from left to right on 4T1, MCF7, SKBR3, MDA-MB-231 cell lines treated with MTP-NeuNT compared to Vehicle (LDS) at 10-7M and 10-6M for 24h. Only cells expressing ErbB2 exhibit reduced proliferation when treated with the therapeutic peptide. Data are presented as mean ± SEM from triplicate experiments. P-values were determined by Mann Whitney test in comparison to control LDS at 10-7M and 10-6M for

24h. *** = P < 0.0001, ** = P < 0,001 compared to vehicle. n.s., not significant. **(C) Decrease of p-Akt and p-Erk phosphorylation.** 72h after addition of MTP-NeuNT at 10^{-6}M on NT193 cells Akt phosphorylation is significantly decreased by 48%, and Erk1/2 phosphorylation is decreased by 15% when evaluated by western blotting. Three independent western-blots were quantified and results are normalized to total Akt or Erk1/2. P-value is determined by Chi-square test. *** = P < 0.0001 compared to vehicle.

Supplemental figure 3:

This figure is supporting figure 3 of the manuscript to demonstrate that the observed therapeutic effect is not correlated with general side effects. This is demonstrated by a detailed examination of kidney and liver at the histological level.



Tolerance of the treatment. (A) H&E staining of liver in vehicle and treated animals (cumulated dose 662µg) demonstrating no sign of tissular lesion when exposed to MTP-NeuNT peptide treatment. (B) H&E staining of kidney in vehicle and treated animals (cumulated dose 462µg) presenting the lack of toxic effect at the histological level after treatment. The analysis was performed under the supervision of a pathologist (Dr Marie-Pierre Chenard, MD) in Hautepierre hospital (CHU Strasbourg) for 10 animals per group.

Supplemental Method – Manuscript by Arpel et al., Cell Reports

Supplemental table 1:

This table is detailing the sequences of peptides analyzed in the BACTH assay described in figure 1.

Peptide	Sequence
MTP-NeuNT / NeuNT	VTFIIATVEGVLLFLILVVVVGILIKRR
mErbB2	VTFIIATVVGVLLFLILVVVVGILIKRR
GpA	IEITLIIFGVMAGVIGTILLISYGIRRK
GpAm	IEITLIIFGVMAIVIGTILLISYGIRRK
VEGFR1	NLELITLCTCVAATLFWLLLTLFIRKK
VEGFR2	IIILVGTAVIAMFFWLLLVIILRTVKRK
VEGFR3	SMEIVILVGTGVIAVFFWVLLLLIFCNRRK
Integrin β-1	IIPIVAGVVAGIVLIGLALLLIWKLLKR
Neuropilin-1	ILITIIAMSALGVLLGAVCGVVLYRKR
EGFR	IIATGMVGALLLLLVVALGIGLFMRRR
hErbB2	ISIISAVVGILLVVVLGVVFGILIKRR
hErbB3	ITMALTVIAGLVVIFMMLGGTFLYWRGRR
hErbB4	IIAAGVIGGLFILVIVGLTFAVYVRRK
cMet	NFTGLIAGVVSISTALLLLLGFFLWLKKR
mEGFR	IATGIVGGLLFIVVVALGIGLFMRRR
mErbB3	VIAVTVGLTVIFLILGGSFLYWRGRR
mErbB4	IAAGVIGGLFILVIMALTFAVYVRRK

Second Objective

VII) To assess whether targeting neuropilin-1 and -2 with membrane targeting peptides would impede breast tumor growth and metastasis

Paper in preparation

Inhibition of triple negative primary breast tumor growth and metastasis using neuropilin-1 transmembrane domain interfering peptide

Alexia Arpel[1,2], Aurore Fernandez[1], Laurent Jacob[1], Nadège Baumlin[1], Patrice Laquerriere[2], Gérard Crémel[1] and Dominique Bagnard[1*]

[1]INSERM U 1109, Labex Medalis, Strasbourg University, Strasbourg, France

[2]CNRS UMR 7178, Institut Pluridisciplinaire Hubert Curien, Strasbourg University, Strasbourg, France

***Corresponding author:**

Dr Dominique Bagnard

INSERM U1109 Université de Strasbourg, Labex Medalis

3 avenue Molière, 67200 Strasbourg, France

Tel : +33-(0)3-88275364 Fax : +33-(0)3-88263538 Email: bagnard@unistra.fr

Running title: New anti-neuropilin peptide inhibits triple negative breast cancer

Keywords: Triple negative breast cancer, metastasis, therapeutic peptide, in vivo model, NRP1, NRP2

Total number of characters: 3143

Summary

The role of transmembrane domains in membrane receptor activation and regulation is nowadays appearing as a key step of cell signaling. Consistently, our team has demonstrated that a peptide targeting the transmembrane domain of neuropilin-1 (MTP-NRP1), blocked cell proliferation, cell migration and angiogenesis *in vitro*, and this lead to the decrease of growth of glioblastoma *in vivo* (Nasarre et al., 2010; Roth et al., 2008). While further characterizing the clinical potential of MTP-NRP1 on breast cancer and metastatic breast cancer, we also decided to extend this novel strategy to the NRP1 closely related membrane receptor NRP2. Mounting evidences support that both receptors are implicated in breast cancer and metastasis processes and are correlated with poor prognosis in such cancer (Stephenson et al., 2002; Bachelder et al., 2003; Yasuoka et al., 2009; Jubb et al., 2012). Our *in vitro* data revealed the blocking activity of both peptides on proliferation on MDA-MB-231 cell line, a triple negative human breast cancer cell line. Moreover, *in vitro* data confirmed the anti-angiogenic effect of MTP-NRP1 and showed that MTP-NRP2 also inhibits angiogenesis. Finally, both peptides were evaluated *in vivo* using an orthotopic breast cancer model and a systemic breast cancer metastasis model. Animals were treated three times a week for three months in both models. While all animals (100%) harboring primary tumor responded to MTP-NRP1 treatment, only 67 % of the MTP-NRP2 treated animals responded to the treatment, as measured using the RECIST criteria (response evaluation criteria in solid tumors)(Therasse et al., 2000). Both treatments induced a significant decrease in total number and extent of breast cancer metastasis. Nevertheless, when analyzing sites of metastasis in details MTP-NRP2 treated animals exhibited a significant increase number of metastasis in bones. Overall, our results report that targeting the TMD of NRP1 receptor in

breast cancer is a potent new strategy to fight against breast cancer and related metastases, while blocking NRP2 may induce unexpected severe side effect in the bone.

Introduction

Since a decade, even though broad achievements were assessed in early breast cancer diagnosis, the death of woman due to breast cancer and related metastasis remains a sobering fact (Lu et al., 2009, Medina, 2005). This indicates the need to develop new strategies and therapeutic tools with effective anti-metastatic properties. To address this need, we tested a novel strategy inhibiting new breast cancer targets: neuropilin-1 and neuropilin-2 protein. Indeed, initially described for their role in nervous system development (Bagri and Tessier-Lavigne, 2002b) and angiogenesis (Jocic and Staton, 1993) for NRP1 and lymphangiogenesis for NRP2 (Yuan et al., 2002), extensive work implicates a role of both receptors in breast cancer tumorigenesis and metastasis. Indeed, besides a role in the survival of breast cancer cells (Bachelder et al., 2001), numerous studies suggest a role of neuropilins in cell migration and metastasis (Fernandis et al., 2004; Yasuoka et al., 2009; Caunt et al., 2008). Moreover, both proteins are expressed in human breast cancer biopsies (Yasuoka et al., 2009; Ghosh et al., 2008; Jubb et al., 2012) and the expression of both proteins is correlated with patient survival (Yasuoka et al., 2009; Ghosh et al., 2008). *In vitro, in vivo* and clinical findings taken together highlights a crucial role for neuropilins in breast cancer tumor growth and metastasis in addition to their involvement in tumor vascularisation. Thus, neuropilins are ideal new attractive targets in breast cancer and some studies have shown this to already be the case *in vitro* and *in vivo*. Barr and co-workers demonstrated in 2005 that a peptide targeting the VEGF165-binding site of NRP1, antagonises the autocrine anti-apoptotic effects of VEGF on 4T1 and MDA-MB-231 breast carcinoma cells *in vitro*. Moreover, another group described that a heptapeptide also inhibiting VEGF binding to NRP1 reduced tumor volume, blood vessel density and endothelial cell area in an orthotopic mammary carcinoma model of MDA-MB-231 cells

(Starzec et al., 2006). Strategies targeting extracellular or intracellular domains of receptors implicated in tumorigenesis are widely accepted and validated, but mounting evidences nowadays demonstrate that the transmembrane domains act as crucial regulators of integral membrane receptor interactions (Hubert et al., 2010). Interestingly, our previous work showed that a peptide mimicking the transmembrane domain of neuropilin-1 (Membrane Targeting Peptide of neuropilin-1 (MTP-NRP1)) exhibit *in vitro* and *in vivo* anti-proliferative, anti-migratory and anti-angiogenic properties (Nasarre et al., 2010; Roth et al., 2008). After successful inhibition of glioma growth, we decided to explore how this strategy would apply for the inhibition of NRP1 and NRP2 in breast cancer and how this may influence the metastatic progression that remains currently a major issue. Hence, we decided to conduct this approach in an animal model of orthotopic breast cancer and in a model of systemic breast cancer metastasis, both mimicking the human pathology. As no targeted therapy is currently available for triple negative breast cancer, the most aggressive breast cancer, *in vivo* experiments were fulfilled grafting MDA-MB-231 (ER-, PR, HER2-). Here, we report that long term tri-weekly intraperitoneal administration of a synthetic Membrane Targeting Peptide (MTP) mimicking the transmembrane domain of neuropilin-1 significantly improved the overall survival of mice compared to vehicle-treated animals (+14% of the mean survival). This benefit could be attributed to reduced primary tumor growth evaluated using both bioluminescence and the RECIST criteria (Eisenhauer et al., 2009), and the reduction of number and burden of systemic metastasis as shown by a longitudinal bioluminescence study. These data are supported by *in vitro* assays including proliferation and angiogenesis assays. However, the results obtained with a peptide targeting NRP2 (MTP-NRP2) revealed that while the peptide significantly decreased the overall burden and number of metastasis, survival was not significantly improved. This could be due to an increase in bone metastasis

occurrence. Overall, our data not only validate that the use of peptides antagonizing NRP1 is a very powerful approach to fight against triple negative breast cancer growth and metastases, but also exemplify that drugs targeting the transmembrane domain of tyrosine kinase receptors offer a convincing alternate to conventional drugs targeting extra or intracellular domains of tyrosine kinase and in general membrane receptors.

Results

MTP-NRP1 and MTP-NRP2 inhibit proliferation of neuropilins-expressing breast tumor cells

To address the biological activity of MTP-NRP1 and MTP-NRP2 synthetic peptides mimicking the TMD of the NRP1 and NRP2, we performed a proliferation MTT assay. This assay was conducted on human MDA-MB-231 triple negative breast cancer cells. The analysis of the expression of both receptors at the RNA or protein levels showed that MDA-MB231 cells expressed NRP1 and NRP2 receptor (figure 1A and 1B). In order to fully characterize the cell phenotype, estrogen, progesterone and HER2 receptors mRNA were analyzed using QPCR to confirm the triple negative phenotype of the cells (figure 1B). Strikingly, as high HER2 mRNA quantity was observed, HER2 protein expression was also checked to definitely confirm the triple negative phenotype of MDA-MB-231 (figure 1A). Cells were treated with a dose response ranging from 10^{-8}M to 10^{-6}M of both peptides for 24 hours. As for the MTP-ErbB2 peptide (Arpel et al., under revision), the peptides were maintained in a non-aggregated form thanks to a buffer containing LDS (lithium dodecyl sulphate). MTP-NRP1 induced a significant reduction of cell numbers from 10^{-8}M (-16% at 10^{-8}M compared to control LDS conditions, $p < 0.0001$ Mann Whitney test respectively), and MTP-NRP2 induced a significant decrease of cell number but only from 10^{-7}M (-19% of cells at 10^{-7}M compared to control LDS conditions, $p < 0.0001$ Mann Whitney test). Taken together, both peptides showed significant inhibition of cell numbers on MDA-MB-231, triple negative breast tumor cells (figure 1C).

MTP-NRP1 and MTP-NRP2 are anti-angiogenic agents

Since neuropilin-1 is highly important in angiogenesis (Jocic and Staton, 1993) and that mounting evidence suggest that neuropilin-2 could also play a role in angiogenesis (in particular through the binding with co-receptors such as VEGFR2 (Pellet-Many et al., 2008)), we evaluated both *in vitro* and *in vivo* how MTP-NRP1 and MTP-NRP2 peptides would interfere with blood vessel formation. First we verified that MTP-NRP1 demonstrated an anti-angiogenic effect using the matrigel assay favoring HUVEC tube formation (Nasarre et al., 2010), which was conducted simultaneously with MTP-NRP2. As expected, the addition of MTP-NRP1 peptide significantly inhibited the tube-like structure within 4h at 10^{-7}M (-38% compared to control condition, $p < 0.0001$, Mann Whitney test), a concentration previously shown to induce a maximal effect in this assay. Interestingly, MTP-NRP2 also exhibited a significant anti-angiogenic effect at 10^{-7}M (-54% compared to control condition, $p < 0.0001$, Mann Whitney test) (figure 2A). Hence, we confirmed the anti-angiogenic effect of both peptides using another widely used angiogenesis model: the in vivo mouse retinal angiogenesis assay. In this case, treating the mice with MTP-NRP1 peptide induced a significant decrease in the distance of vascular front migration (8% at 1,5 μg/Kg, $p < 0.0001$, Mann Withney test). MTP-NRP2 also induced a significant inhibition (-3%, $p = 0.0215$, Mann Whitney test) of the vascular front migration although being less pronounced that the one of MTP-NRP1 (figure 2B). Thus, these results confirmed the anti-angiogenic properties of MTP-NRP1 and showed that MTP-NRP2 also inhibits angiogenesis.

MTP-NRP1 and MTP-NRP2 reduce primary breast tumor growth in vivo

We further explored the therapeutic potential of MTP-NRP1 or MTP-NRP2 in a model of orthotopic breast cancer. We decided to monitor tumor growth by injecting luciferase expressing MDA-MB-231 cells to easily determine tumor extent using bioluminescence quantitation at week 2, 5, 9 and 13 post grafting using the NightOwl bioimager apparatus. Initial experimental conditions were standardized by establishing groups of similar cumulated bioluminescence two weeks after grafting 10^6 cells in the mouse mammary fat pad (average bioluminescence in the vehicle group being 4147 cps, 4189 cps in the MTP-NRP1 group ($p = 0.9$ Mann Whitney), and 3469 cps in the MTP-NRP2 group ($p = 0.2$ compared to control, Mann Whitney test). Mice were treated three times a week by intra-peritoneal injection of either the vehicle (LDS, 72 μM), MTP-NRP1 or MTP-NRP2 at 10^{-7}M, a concentration previously shown to induce a significant decrease of MDA-MB-231 cell proliferation in *in vitro* experiments for both peptides and to provoke a decrease of glioma growth *in vivo* for MTP-NRP1 peptide (Nasarre et al., 2010). A total number of 15 animals composed each group. Figure 3A is presenting representative examples of the orthotopic tumors detected in the control, the MTP-NRP1 and in the MTP-NRP2 treated groups. To analyze extensively the response of each animal to the treatments a waterfall plot of best response was assessed for MTP-NRP1 and MTP-NRP2 treatment (determined between the bioluminescence of week 13 and week 9 according to the RECIST criteria). This analysis revealed that 100% of the MTP-NRP1 treated animals responded to the treatment with 25% of SD (Stable Disease, < -30% decrease of target lesion) and 75% with PR (Partial Response > 30% decrease of target lesion) including two individuals above or equal to 90% of decrease in tumor volume (figure 3B). Noteworthy, 67 % of the MTP-NRP2 treated animals responded to the treatment (17% of stable disease and 50 % of partial response) but 33% of the animals

showed tumor progression (increase above 20% of target lesion)(figure 3B). This part of the study demonstrated that blocking NRP1 in triple-negative breast tumor significantly block tumor growth for all treated animals. However, one third of the animals were not responding to a treatment inhibiting NRP2.

MTP-NRP1 and MTP-NRP2 exhibit anti-metastatic properties

The occurrence of lung metastasis is a crucial step in breast cancer progression and linked to disease-associated death (Chambers et al., 2002). Thus, we decided to monitor whether MTP-NRP1 and MTP-NRP2 would impede breast cancer metastasis progression. Animals were here again treated with 10^{-7}M of MTP-NRP1 or MTP-NRP2 peptide or with the vehicle (LDS, 72 μM). Treatment started 2 days post-intracardiac grafting of 10^5 cells in the left ventricle. Figure 4A is exhibiting representative examples of mice developing metastases over time in the three experimental groups. Strikingly, while the quantification of cumulated bioluminescent signal showed that the two peptides dramatically reduced metastasis growth (figure 4B), total number of metastasis was significantly reduced in both groups but in a wider extent in MTP-NRP1 treated animals (-15% MTP-NRP2 treated animals and -62% in MTP-NRP1 treated animals)(figure 4C). Strikingly, when further detailing the site of metastasis soiling (figure 4D) the number of metastasis decreased significantly in all sites with the exception of a significant marked increase of metastasis number in the bones in the MTP-NRP2 treated group. Hence, while bioluminescence analysis revealed a significant anti-metastatic effect of MTP-NRP1 and MTP-NRP2 characterized by both a reduction of the extent and total number of the lesions, MTP-NRP2 triggered an unexpected increase of bones metastasis.

MTP-NRP1 improves overall survival but not MTP-NRP2

To finally address the therapeutic benefit of MTP peptides we also monitored mice survival during the whole protocol. Interestingly, even though MTP-NRP2 animals exhibit a significant general decrease in the metastasis growth, the Kaplan Meier curve with the log rank test to compare the different groups of treatments revealed that survival was not significantly increased with MTP-NRP2 (81 days of mean survival compared to 75 days in the control group ; $p = 0.3456$ compared to control, Log-ranked test). This could be consistent with the increased number of bone metastases and the existence of quite significant number of mice not responding to this treatment. In contrast, the high response rate of animals to MTP-NRP1, both for primary tumor and in metastasis, translated into a marked and significant survival benefit (+ 14% of the mean survival, $p = 0.0109$ compared to vehicle, Log-ranked test) (figure 5).

MTP-NRP1 and MTP-NRP2 are well-tolerated drugs

Preclinical evaluation of drug toxicity is a mandatory prerequisite in the “go, no-go” path of preclinical validation of a drug. Drug safety testing includes determination of acute and chronic toxicity. This long term three weekly injections by IP administration gave us the unique possibility to evaluate chronic toxicity of the MTP-NRP1 peptide. As MTP-NRP2 peptide exhibited rather pro-metastatic properties in bones, the first metastatic site developed in human breast cancer (Solomayer et al., 2000; Berman et al., 2013) blood samples analysis focused on MTP-NRP1 peptide. Hence blood samples were collected from the cardiac cavity for analysis before sacrifice of animals of both the orthotopic and the systemic metastasis model. In all cases, MTP-NRP1 did not worsen biological parameters of mice including renal, hepatic and heart markers. A seen in table 1, we rather observed a

significant decrease of LDH (reflecting better function of organs) in the treated group. Thus, the long term administration of the MTP-NRP1 peptide is well tolerated.

Discussion

As described by Weinberg (2011) cancer cells exhibit during tumorigenesis sustained proliferation capabilities, present strong migration capacity to invade adjacent tissue and can promote angiogenesis. Here we show that MTP-NRP1 peptide interferes with all of these three key hallmarks of cancer. The observed reduction of primary tumor and the marked reduction in the metastasis extent could relate to both a decrease in breast cancer cell proliferation and angiogenesis. Indeed we have shown the anti-proliferative property of MTP-NRP1 on the MDA-MB-231 at 10^{-7}M the concentration used *in vivo*. Moreover the anti-angiogenic potential of this peptide has been validated on two different models (matrigel assay and mouse retinal angiogenesis assay). In both case, we demonstrate a lack of tube formation and a decrease in the distance of the vascular front migration. Besides, our previous work also demonstrates that MTP-NRP1 is able to decrease endothelial cell proliferation (Nasarre et al., 2010). This could be supported by further analysis of a proliferation marker such as Ki67 and a blood vessel marker such as CD31 or isolectin-B4 on primary tumor histological sections. Interestingly, MTP-NRP1 treatment induces a significant decrease of the number of metastasis. Albeit MTP-NRP2 showed an evident improvement in metastasis extent, no decrease in survival was observed. Rather, our detailed analysis of the sites of metastasis revealed an increase of bone metastasis in animals treated with MTP-NRP2. Interestingly, NRP2 receptor has been linked with bone homeostasis. Indeed, according to Verlinden and collaborators (Verlinden et al., 2013), NRP2 knockout mice exhibited reduced bone mass suggesting a decrease in osteoblast activity, along with an

improved number of osteoclasts and a reduced osteoblast count. The addition of NRP2 inhibiting peptide may therefore lead to the inhibition of osteoblast, favoring bone reduction further strengthened by metastatic cells colonizing bone. Importantly as bones are the primary metastatic site in human breast cancer (Solomayer et al., 2000; Berman et al., 2013), our results identified a major drawback for MTP-NRP2 treatment as demonstrating a high risk of severe adverse effect in the form of additional bone metastasis. Therefore, in depth analyses are required to explore the multifaceted relationship of the various factors controlling neuropilin-2 signaling platform in breast tumor development. Here we obtained the anti-tumor effect of MTP-NRP1 with very low dosage of 10^{-7}M. This is in accordance with our previous work that showed successful inhibition of glioma growth *in vivo* with the same concentration of the peptide. However, in the case of breast cancer cells, the maximal effect was obtained with 10^{-6}M (*in vitro* corresponding dosage to 1.5 μg/kg), a concentration similar to the one employed for MTP-NeuNT peptide targeting the TMD of NeuNT receptor. It would be interesting to evaluate higher concentration of MTP-NRP1 to check whether we already reached the maximal anti-tumor effect. Importantly, MTP-NRP1 peptide showed no toxicity according to blood analysis after a long period of treatment therefore suggesting that we had not reached yet the maximal tolerated dose. This was already the case in previous assays using MTP-NRP1 peptide (Nasarre et al., 2010) and was also observed when analyzing liver and kidney sections of mice under MTP-NeuNT peptide treatment (Arpel et al., 2013, under revision). Noteworthy, no cutaneous lesions were observed on all animals and during each protocol around the intra-peritoneal injection site of the different drugs.

Conclusive remarks

Targeting the transmembrane domain of bitopic receptors such as NRP1 provides a new exquisite therapeutic tool. Previous work demonstrated a crucial role of the TMD of NRP1 and suggested that inhibition may represent a therapeutic potential in glioma treatment (Roth et al., 2008; Nasarre et al., 2010). This strategy has been now validated on another type of cancer: breast cancer and related metastases. Our results clearly revealed that peptide mimicking the transmembrane domain of NRP1 decreased both the extent and the number of breast metastasis translating into a marked improvement of survival. This is supported by anti-proliferative and anti-angiogenic properties *in vitro*. Importantly, this therapeutic benefit was achieved using micromolar dosage and no toxicity was observed according to blood analysis. In contrast to MTP-NRP1 peptide, for MTP-NRP2 peptide, future investigation has to be fulfilled to fully understand its effect. Here we report that targeting the TMD of NRP1, with a peptide mimicking its transmembrane domain could be a future potent drug in breast cancer. Future investigation will evaluate this unique strategy on other tyrosine kinase receptors or other single spanning membrane proteins with key functions in the process of cancer growth.

Material and methods

Cell Culture

The human epithelial breast adenocarcinoma derived from pleural effusion MDA-MB-231 (ER-, PR-, HER2-), MCF7 (ER+, PR+, HER2+), SKBR3 (ER-, PR-, HER2+) cells were grown in Dulbecco's modified Eagle medium (DMEM, GIBCO) and HUVEC cells were cultured in Endothelial Cell Growth Medium (Promo Cell). MDA-MB-231 cells were purchased from ECACC (92020424), HUVEC cells were purchased from Promo Cell (C-12200), MCF7 and SKBR3 cells were obtained from our institute collection. All cell mediums were supplemented with 10% fetal calf serum (FCS) (GIBCO), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma), and cultured at 37ºC, 5% CO_2. HUVEC cells were cultured with added supplements: ECGS (0.004 ml/ml), hEGF (0.1 ng/ml), hbFGF (1 ng/ml) and FCS (0.02 ml/ml). Cultures were grown to 70 to 80% confluence (not grown over 100% confluency) and were routinely split in 10 cm culture dishes. Culture were suspended with trypsin-EDTA (0.05 % trypsine, 0.02 % EDTA), spin down and split regularly up to 40 passages before new stocks were thawed with the exception of HUVEC cells split up to 5 passages maximum.

Peptides

Peptides have been synthesized by Peptide Specialty Laboratories GmbH using automatic peptide synthesis (Fmoc chemistry). The peptide corresponding to the TM sequence of NRP1: ILITIIAMSALGVLLGAVCGVVLYRKR is referred as MTP-NRP1, the peptide corresponding to the TM sequence of NRP2: ILITIIAMSSLGVLLGATCAGLLLYRKR is referred as MTP-NRP2. Peptides purity estimated by RP-HPLC was more than 95% according to manufacturer indication.

RTQ-QPCR

RNA was extracted with TriReagent solution according to manufacturer's instruction (Molecular Research Center Inc., Euromedex). RNA was treated with DNaseI (Invitrogen) and reverse transcribed using the High Capacity cDNA RT Kit (Life Technologies). Quantitative reverse transcriptase polymerase chain reaction (RTQ-PCR) was performed using the Power SYBR Green PCR Master Mix or TaqMan Gene Expression Master Mix (Life Technologies) using the 7500 Real time PCR System (Life Technologies) following the manufacturer's protocol. SYBER green NRP1 sens 5'- CCCGAGAGAGCCACTCATG-3'; NRP1 anti-sens 5'-GTCATCACATTCATCCACCAA-3'; NRP2 sens 5'- CAATTGCAACTTCGATTTCCTC-3'; NRP2 anti-sens 5'-CCGGTCGTTTGG GCTGGA-3'; ER sens 5'- AGCCCGCTCATGATCAAACGCTC-3'; ER anti-sens 5'-GAATAGAGTATCGGGGGCTC-3'; PR sens 5'- TTCCCGTTGGGGCCACCGCCC-3'; PR anti-sens 5'-ATGCACTCCAGGGTCGACCCCG-3'; HER2 sens 5'- CAATGGAGACCCGCTGAAC-3'; HER2 anti-sens 5'-CAGTGCGCGTCAGGCTCT-3' primers were used to quantify human NRP1, NRP2, ER, PR and HER2 mRNA. Samples were analyzed using 2 µl cDNA. All data were normalized to the GAPDH human reference gene using Taqman GAPDH probe (GAPDH-Hs99999905_m1). For the evaluation of human receptors RNA quantity in the breast cancer cells, calculation were effectuated as the following: Δct(MDA-MB-231$_{NRP1}$) = ct (NRP1) – ct (GAPDH), mRNA quantity = 2^(-Δct(MCF7$_{NRP1}$)).

Immunocytochemistry

Cells were grown on sterile glass cover slips for one day before immunofluorescence staining. The cells were fixed with freshly made fixative 4% formaldehyde (FA) for 10 minutes. The samples were gently rinsed with PBS (1 wash for 10 minutes) before adding the blocking solution (FCS 5%) for a minimum of 30 minutes. Cells were permeabilized using *1x*

PBS with 0.1% Tween20 for 5 minutes. Appropriate primary anti-body (anti-human anti-body to rabbit neuropilin-1 (sc-5541; Santa Cruz Biotechnology) and anti-human anti-body to rabbit neuropilin-2 (H-300) (sc-5542; Santa Cruz Biotechnology) antibody diluted in 5% fetal calf serum (FBS)-PBS was added to the cell over night at room temperature. After thorough wash (3 washes of 5 minutes) appropriate secondary antibody (goat anti-rabbit, Nordic Immunology GAM/Fab/TRITC diluted 1/1000 from stock) was added at room temperature for 90 minutes. After washing in PBS, the cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole, 1/30 000 in water) for 10 minutes. Glass coverslips were finally mounted on microscopy glass slides using a polymerization medium (FluorSave reagent, Calbiochem-Merck cat#345789).

Cell proliferation

In vitro cell proliferation was monitored using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) proliferation assay according to manufacturer's instruction (Sigma, M2128, USA). Optical density was determined at 570 nm using an ELISA plate reader spectrophotometer (EL800, Bio-Tek Instruments). Appropriate cells were seeded at a density of 10 000 cells per well in a 96 well plate, the cells were then incubated with increasing peptide concentration (ranging from 10^{-9}M to 10^{-6}M) or corresponding vehicle increasing concentration (LDS, ranging from 0.72 µM to 720 µM). After 24h incubation period the culture media were removed from the well and 100 µl of MTT dye freshly diluted (to 1/50 in GBSS) from stock solution (5 mg/ml) was added to each well for 4h. After this incubation period, isopropanol (100 µl) was added to the MTT solution in each well before reading the optical density at 570 nm. For cell fluorescence acquisition, images were acquired with the fluorescence Zeiss Imager Z2 equipped with HXP 120W lamp and ApoTome system.

HUVEC tube-like formation assay

The tube-like formation assay was performed using manufacturer's instruction (*In vitro* angiogenesis Assay kit; Millipore). Briefly the extracellular matrix (ECM) matrigel and diluent were pre-chilled at 4°C. On a 96 well plate 70µl of a solution composed of 10% of ECM diluent and 90% of ECM matrix solution was deposited per well. This matrigel solution was then allowed to polymerise for 1 hour at 37°C. HUVEC (from 70% confluent dishes grown after a maximum of 5 passages) were trypsinised, pelleted and 5000 cells were seeded in each well. After adding the desired compound concentration, the plates were incubated for a period of 4 hours allowing tube-like structures formation. The number of tube-like structures was quantified using the Image J software from four pictures per condition. For each compound tested at least three independent assays with triplicates per condition were performed.

The mouse retinal angiogenesis assay

C56Bl/6 mice were treated on a daily basis with subcutaneous injection of desired drug (100 µl) from P2 up to P5. Freshly enucleated eyes from the P5 mice were fixed in 4% formaldehyde for 1 hour on ice. Retinas were dissected out of the eyes after careful removal of lenses. Then retinas were stained overnight at 4°C with FITC-isolectin B4 (Vector) a marker for vessels. Retina were then flattened and mounted with Vectashield medium. Retina cell fluorescence images were acquired with the fluorescence Zeiss ImagerZ2 using the mosaic mode. For quantification, the distance of the vascular front migration from the optic nerve was measured at 8 different regions covering the whole retinae for each specimen. All lengths were normalized to control (100%) of each experiment. For each compound a minimum of three P5 mice from three litters were evaluated.

Orthotropic grafting of cells in the mouse mammary Fat Pad

Nude mice (8 weeks of age) were anesthetized (Initially 3% isoflurane with air/O_2 mix, then the animal was kept under anaesthesia with 1.5% Isoflurane). A small incision up to the sternum was completed, and an angled lateral incision from the initial small incision was made towards a leg. The blood vessel emerging between these fat pads was cauterised (Electric cauterizer (FST No 18000-00)). Then the needle (BD Microlance; 22G ¼ - Nr 12; 0,7 x 30 mm, REF 300900) on the syringe containing 10^6 cells in 50 μl of PBS was inserted in the mammary fat pad from the external side up to the lymph node and the cells were injected behind the lymph node within the mammary fat pad. Skins were then aligned as closely to each other as possible and sutured. The animal was monitored until it was awakened from the procedure and was moving around the cage normally and then observed on a daily basis until sacrifice. For bioluminescence detection, IP injection of 100 μl of a luciferin solution at 30 mg/ml was completed on a weekly basis for each mouse. Acquisition was operated for 5 min (filter 560 nm) using a live imager (NightOwl system Berthold).

Intracardiac injection

Cells detached with Versene (an EDTA solution used in order to assess a gentle non-enzymatic cell dissociation), washed and counted for 10^5 in 100 μl of PBS, and were injected into the left ventricle of anesthetized (Initially 3% isoflurane with air/O_2 mix, then the animal was kept under anaesthesia with 1.5% isoflurane) nude mice (8 weeks old) using a 26G ½ needle and a 1 ml syringe. Blood should enter the needle with pulsatile pressure and light red colour to be sure of not being in the right ventricle were blood is darker because of oxygen depletion. Anaesthesia and post-surgical animal monitoring procedure, and bioluminescence detection were identical to orthotopic injection.

Animal handling and in vivo ethical statement

Experiments were performed according to the Guide for Care and Use of Laboratory Animals (E67-6-482-21) and the European Directive with approval of the regional ethical committee (Reference AL/55/62/02/13). Mice received food and water ad libitum. Animals were sacrificed using CO_2. All necessary precautions were taken to minimize pain or discomfort of the animals. General health status was monitored 3 times a week by independent observers. Sacrifice of the animal was effectuated when reaching limit ethical endpoints.

Statistics

Statistical analyses were performed using Mann Whitney test (for sample n < 30), Log ranked test for survival analysis using GraphPad software (USA). P-values are given in the figure legends, and values of $P < 0.05$ were considered to be statistically significant. Normal distribution of the values was checked using GraphPad software (USA). A minimum of three independent experiments was performed for *in vitro* assays (proliferation, angiogenesis). For *in vivo* experiment sample size calculation anticipated a therapeutic effect of 20% for a standard deviation of 14% and confidence interval of confidence 95% (Lamorte's Power calculation, University of Boston).

References

Bachelder, R.E., A. Crago, J. Chung, M.A. Wendt, L.M. Shaw, G. Robinson, and A.M. Mercurio. 2001. Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. *Cancer Res.* 61:5736–5740.

Bachelder, R.E., E.A. Lipscomb, X. Lin, M.A. Wendt, N.H. Chadborn, B.J. Eickholt, and A.M. Mercurio. 2003. Competing autocrine pathways involving alternative neuropilin-1 ligands regulate chemotaxis of carcinoma cells. *Cancer Res.* 63:5230–5233.

Bagri, A., and M. Tessier-Lavigne. 2002. Neuropilins as Semaphorin receptors: in vivo functions in neuronal cell migration and axon guidance. *Adv. Exp. Med. Biol.* 515:13–31.

Berman, A.T., A.D. Thukral, W.-T. Hwang, L.J. Solin, and N. Vapiwala. 2013. Incidence and patterns of distant metastases for patients with early-stage breast cancer after breast conservation treatment. *Clin. Breast Cancer*. 13:88–94. doi:10.1016/j.clbc.2012.11.001.

Caunt, M., J. Mak, W.-C. Liang, S. Stawicki, Q. Pan, R.K. Tong, J. Kowalski, C. Ho, H.B. Reslan, J. Ross, L. Berry, I. Kasman, C. Zlot, Z. Cheng, J. Le Couter, E.H. Filvaroff, G. Plowman, F. Peale, D. French, R. Carano, A.W. Koch, Y. Wu, R.J. Watts, M. Tessier-Lavigne, and A. Bagri. 2008. Blocking neuropilin-2 function inhibits tumor cell metastasis. *Cancer Cell*. 13:331–342. doi:10.1016/j.ccr.2008.01.029.

Chambers, A.F., A.C. Groom, and I.C. MacDonald. 2002. Dissemination and growth of cancer cells in metastatic sites. *Nat. Rev. Cancer*. 2:563–572. doi:10.1038/nrc865.

Eisenhauer, E.A., P. Therasse, J. Bogaerts, L.H. Schwartz, D. Sargent, R. Ford, J. Dancey, S. Arbuck, S. Gwyther, M. Mooney, L. Rubinstein, L. Shankar, L. Dodd, R. Kaplan, D. Lacombe, and J. Verweij. 2009. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur. J. Cancer Oxf. Engl. 1990*. 45:228–247. doi:10.1016/j.ejca.2008.10.026.

Fernandis, A.Z., A. Prasad, H. Band, R. Klösel, and R.K. Ganju. 2004. Regulation of CXCR4-mediated chemotaxis and chemoinvasion of breast cancer cells. *Oncogene*. 23:157–167. doi:10.1038/sj.onc.1206910.

Ghosh, S., C.A.W. Sullivan, M.P. Zerkowski, A.M. Molinaro, D.L. Rimm, R.L. Camp, and G.G. Chung. 2008. High levels of vascular endothelial growth factor and its receptors (VEGFR-1, VEGFR-2, neuropilin-1) are associated with worse outcome in breast cancer. *Hum. Pathol.* 39:1835–1843. doi:10.1016/j.humpath.2008.06.004.

Hubert, P., P. Sawma, J.-P. Duneau, J. Khao, J. Hénin, D. Bagnard, and J. Sturgis. 2010. Single-spanning transmembrane domains in cell growth and cell-cell interactions: More than meets the eye? *Cell Adhes. Migr.* 4:313–324. doi:10.4161/cam.4.2.12430.

Jocic, Z., and R.D. Staton. 1993. Reduplication after right middle cerebral artery infarction. *Brain Cogn.* 23:222–230. doi:10.1006/brcg.1993.1056.

Jubb, A.M., L.A. Strickland, S.D. Liu, J. Mak, M. Schmidt, and H. Koeppen. 2012. Neuropilin-1 expression in cancer and development. *J. Pathol.* 226:50–60. doi:10.1002/path.2989.

Nasarre, C., M. Roth, L. Jacob, L. Roth, E. Koncina, A. Thien, G. Labourdette, P. Poulet, P. Hubert, G. Crémel, G. Roussel, D. Aunis, and D. Bagnard. 2010. Peptide-based interference of the transmembrane domain of neuropilin-1 inhibits glioma growth in vivo. *Oncogene*. 29:2381–2392. doi:10.1038/onc.2010.9.

Pellet-Many, C., P. Frankel, H. Jia, and I. Zachary. 2008. Neuropilins: structure, function and role in disease. *Biochem. J.* 411:211–226. doi:10.1042/BJ20071639.

Roth, L., C. Nasarre, S. Dirrig-Grosch, D. Aunis, G. Crémel, P. Hubert, and D. Bagnard. 2008. Transmembrane domain interactions control biological functions of neuropilin-1. *Mol. Biol. Cell*. 19:646–654. doi:10.1091/mbc.E07-06-0625.

Solomayer, E.F., I.J. Diel, G.C. Meyberg, C. Gollan, and G. Bastert. 2000. Metastatic breast cancer: clinical course, prognosis and therapy related to the first site of metastasis. *Breast Cancer Res. Treat.* 59:271–278.

Starzec, A., R. Vassy, A. Martin, M. Lecouvey, M. Di Benedetto, M. Crépin, and G.Y. Perret. 2006. Antiangiogenic and antitumor activities of peptide inhibiting the vascular endothelial growth factor binding to neuropilin-1. *Life Sci.* 79:2370–2381. doi:10.1016/j.lfs.2006.08.005.

Stephenson, J.M., S. Banerjee, N.K. Saxena, R. Cherian, and S.K. Banerjee. 2002. Neuropilin-1 is differentially expressed in myoepithelial cells and vascular smooth muscle cells in preneoplastic and neoplastic

human breast: a possible marker for the progression of breast cancer. *Int. J. Cancer J. Int. Cancer*. 101:409–414. doi:10.1002/ijc.10611.

Therasse, P., S.G. Arbuck, E.A. Eisenhauer, J. Wanders, R.S. Kaplan, L. Rubinstein, J. Verweij, M. Van Glabbeke, A.T. van Oosterom, M.C. Christian, and S.G. Gwyther. 2000. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J. Natl. Cancer Inst.* 92:205–216.

Verlinden, L., C. Kriebitzsch, I. Beullens, B.K. Tan, G. Carmeliet, and A. Verstuyf. 2013. Nrp2 deficiency leads to trabecular bone loss and is accompanied by enhanced osteoclast and reduced osteoblast numbers. *Bone*. 55:465–475. doi:10.1016/j.bone.2013.03.023.

Yasuoka, H., R. Kodama, M. Tsujimoto, K. Yoshidome, H. Akamatsu, M. Nakahara, M. Inagaki, T. Sanke, and Y. Nakamura. 2009. Neuropilin-2 expression in breast cancer: correlation with lymph node metastasis, poor prognosis, and regulation of CXCR4 expression. *BMC Cancer*. 9:220. doi:10.1186/1471-2407-9-220.

Yuan, L., D. Moyon, L. Pardanaud, C. Bréant, M.J. Karkkainen, K. Alitalo, and A. Eichmann. 2002. Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Dev. Camb. Engl.* 129:4797–4806.

Figure legends

Figure 1: *In vitro* cell characterization and properties of MTP-NRP1 and MTP-NRP2 on MDA-MB-231.

(A) Demonstration of the absence of HER2 protein and the presence of NRP1 and NRP2 receptor at protein level on MDA-MB-231 cells. (B) Q-PCR mRNA quantity of GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), housekeeping gene, NRP1 (neuropilin-1), NRP2 (neuropilin-2), ER (estrogen receptor), PR (progesterone receptor), and HER2 (human epidermal growth factor receptor 2). (C) Demonstration of the dose-dependent anti-proliferative effect of MTP-NRP1 and MTP-NRP2 peptides on MDA-MB-231 cells. ns : not significant, ** $p < 0.01$, *** $p < 0.001$ using Mann and Whitney test.

Figure 2: Anti-angiogenic properties of MTP-NRP1 and MTP-NRP1

(A) Evidence of peptide induced *in vitro* inhibition of angiogenesis using the tube formation assay using HUVECs treated for 4h with MTP-NRP1 and MTP-NRP2 at 10^{-7}M. (B) Confirmation of anti-angiogenic properties *In vivo* using the mouse retinal angiogenesis model. P5 analysis of distance of front migration (%) when animals are daily treated with 1,5µg/Kg of MTP-NRP1 or MTP-NRP2 from P0 to P5. ** $p < 0.01$, *** $p < 0.001$ using Mann and Whitney test.

Figure 3: MTP-NRP1 and MTp-NRP2 reduced primary breast tumor growth *in vivo*

(A) Representative examples of the orthotopic tumor detected in the control, the MTP-NRP1 and in the MTP-NRP2 treated groups with 1,5µg/Kg three times a week over time (2, 5, 9 and 13 weeks). (B) Demonstration of the inhibitory effect of MTp-NRP1 and MTp-NRP2 peptides on primary tumors volumes between week 9 and 13 of treatment. The Waterfall graph represents the percent change in tumor volume of individual treated animals (grey bars, n =

12) compared to the averaged tumor volume increased determined in the control group (dark bar, n = 12). This demonstrates that 100% of the treated animals responded to MTP-NRP1 treatment and 67 % of the treated animals responded to the MTP-NRP2 treatment.

Figure 4: MTP-NRP1 and MTP-NRP2 exhibit anti-metastatic properties

(A) Representative examples of the metastasis detected in the control, the MTP-NRP1 and in the MTP-NRP2 treated groups over time (4, 6, 8, 10, 12 and 13 weeks) treated three times a week at 1,5µg/Kg. (B) Cumulated bioluminescence signal in control, MTP-NRP1 and MTP-NRP2 treated groups over time. (C) Cumulated number of metastasis in entire animals over time. (D) Detailed analysis of cumulated number of metastasis in (a) bone, (b) lung, (c) brain and (d) other "sites" in the mice of control, MTP-NRP1 and MTP-NRP2 treated animals. ** $p < 0.01$, *** $p < 0.001$ using Mann and Whitney test.

Figure 5: MTP-NRP1 improves overall survival but not MTP-NRP2

Kaplan Meyer survival curve demonstrating a significant increased survival of the treated MTP-NRP1 animals compared to the control animals, a beneficial effect not observed with MTP-NRP2. * $p < 0.05$ using Mann and Whitney test.

Table 1: Biochemistry analysis of blood samples of animals under MTP-NRP1 treatment

LDH: Lactate dehydrogenase; ASAT: aspartate transaminase; ALAT alanine transaminase; ALP alkaline phosphatase; Creat: creatinine. ns: not significant, ** $p < 0.01$ using Mann and Whitney test.

Table 2: Numeration analysis of blood samples of animals under MTP-NRP1 treatment

HGB: haemoglobin; HCT: hematocrite; PLT: platelets; Baso: basophil; Eosino: eosinophil; Mono: monocyte; lympho: lymphocyte. ns: not significant, * $p < 0.05$ using Mann and Whitney test.

Figure 1: *In vitro* cell characterization of MDA-MB-231 and *in vitro* properties of MTP-NRP1 and MTP-NRP2 on MDA-MB-231

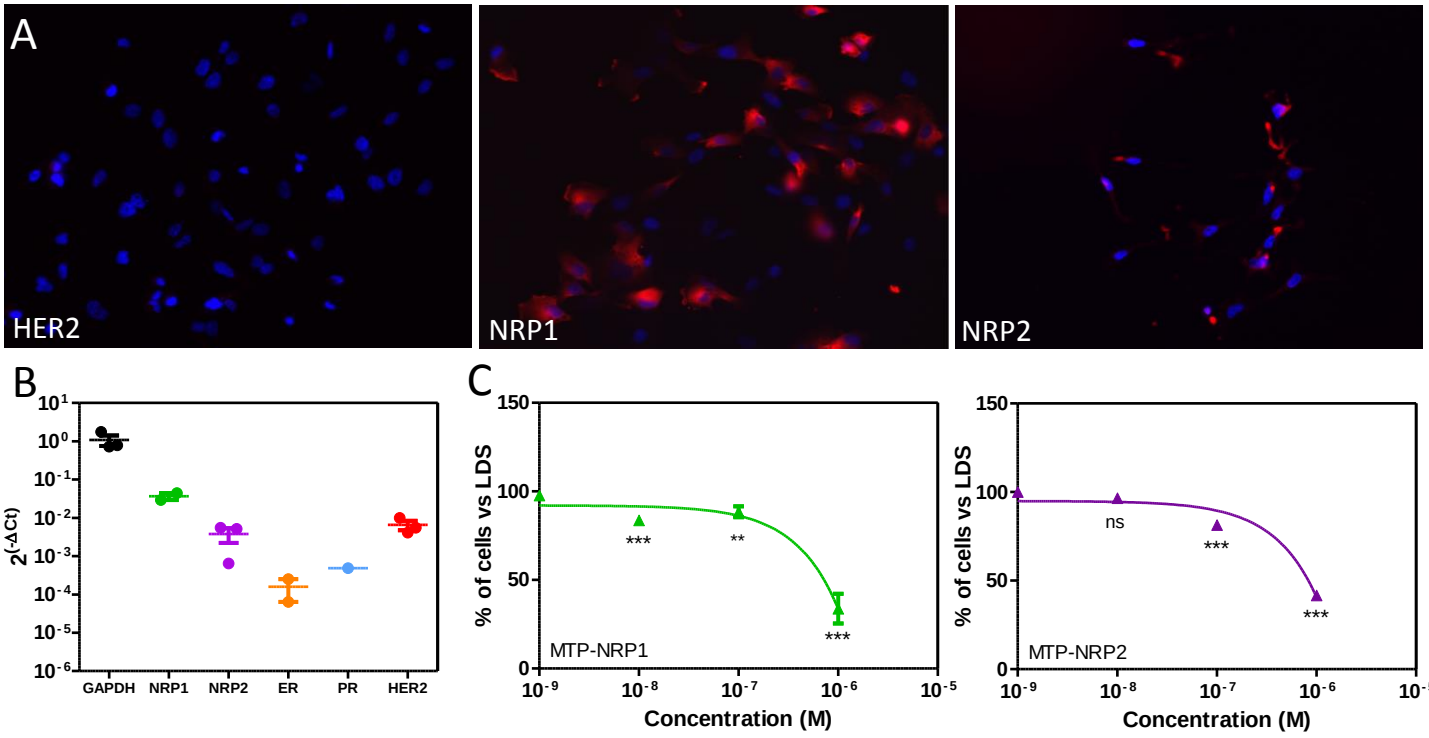


Figure 2: Anti-angiogenic properties of MTP-NRP1 and MTP-NRP2

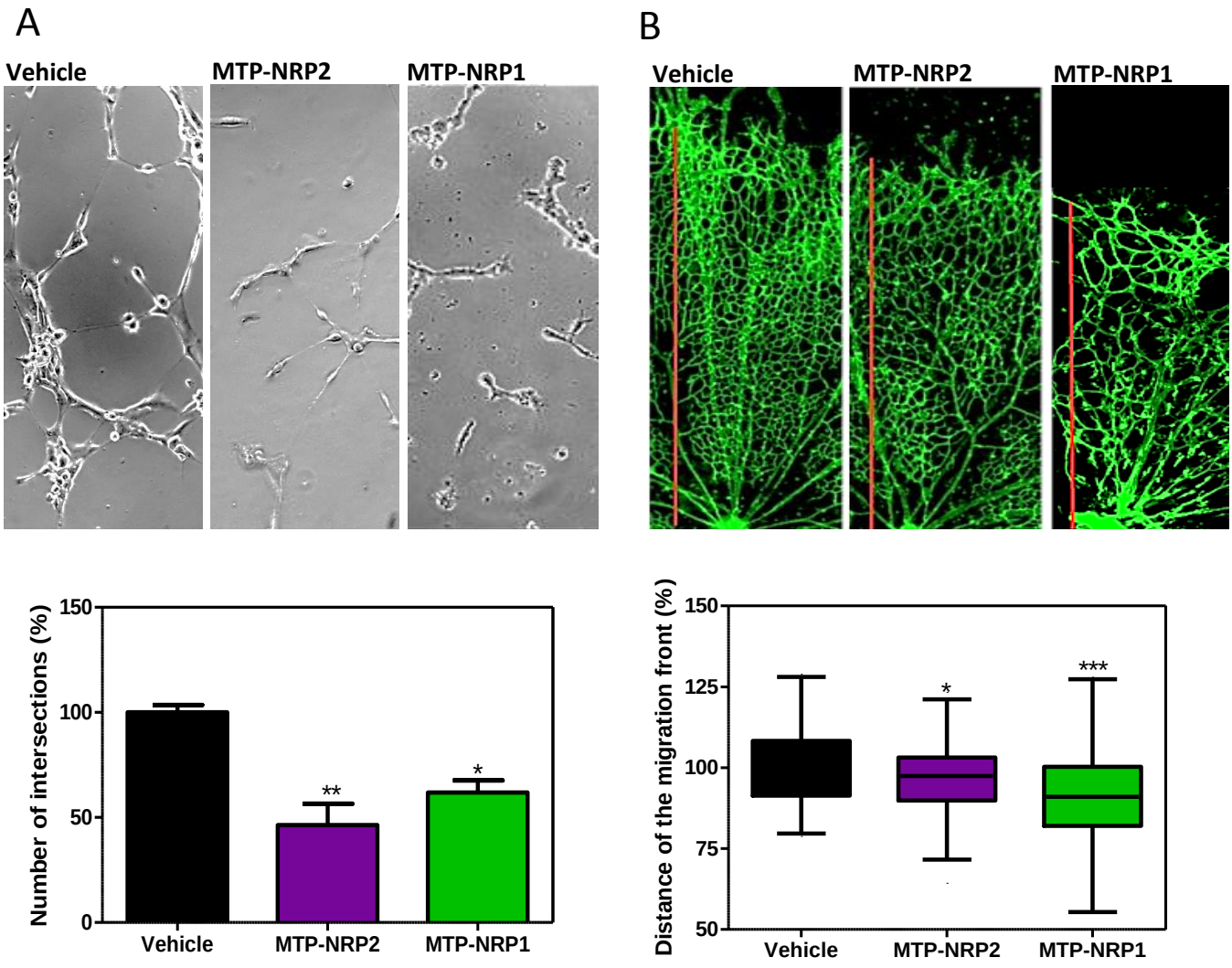


Figure 3: MTP-NRP1 and MTp-NRP2 reduced primary breast tumor growth *in vivo*

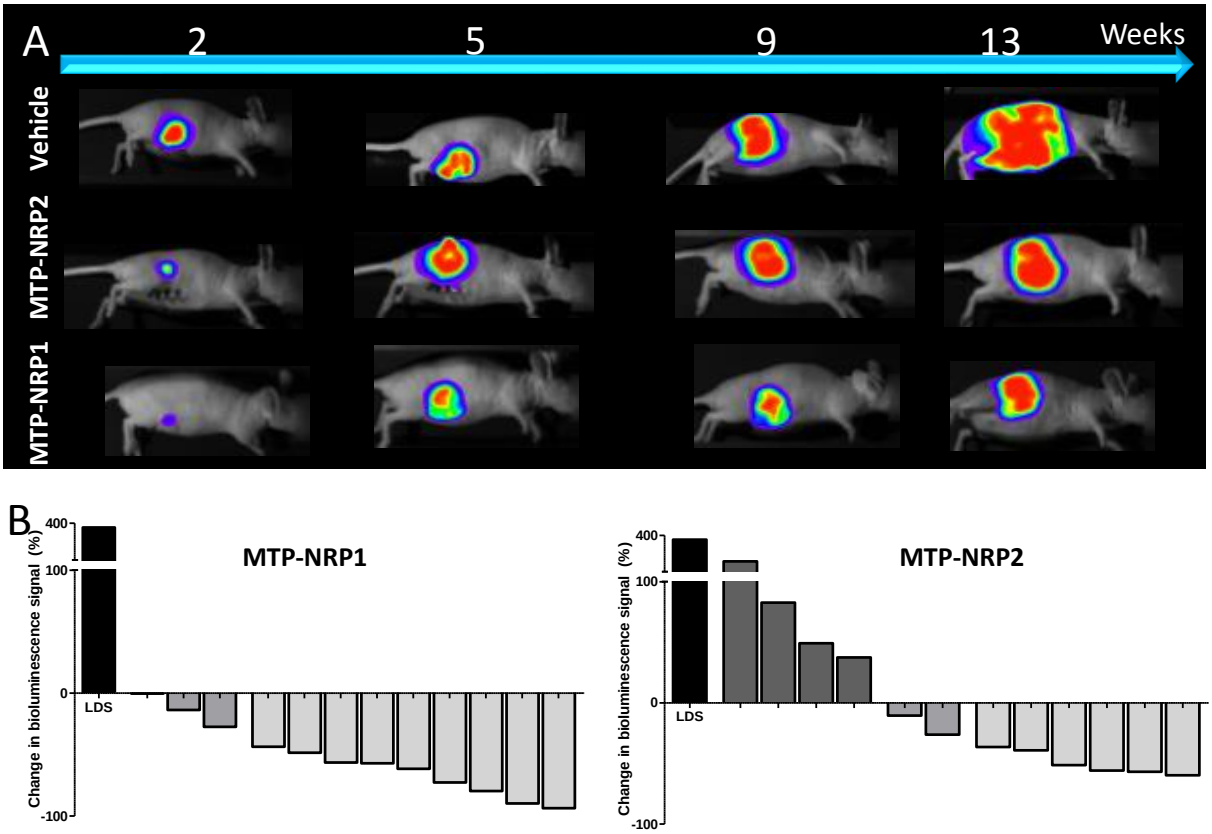


Figure 4: MTP-NRP1 and MTP-NRP2 exhibit anti-metastatic properties

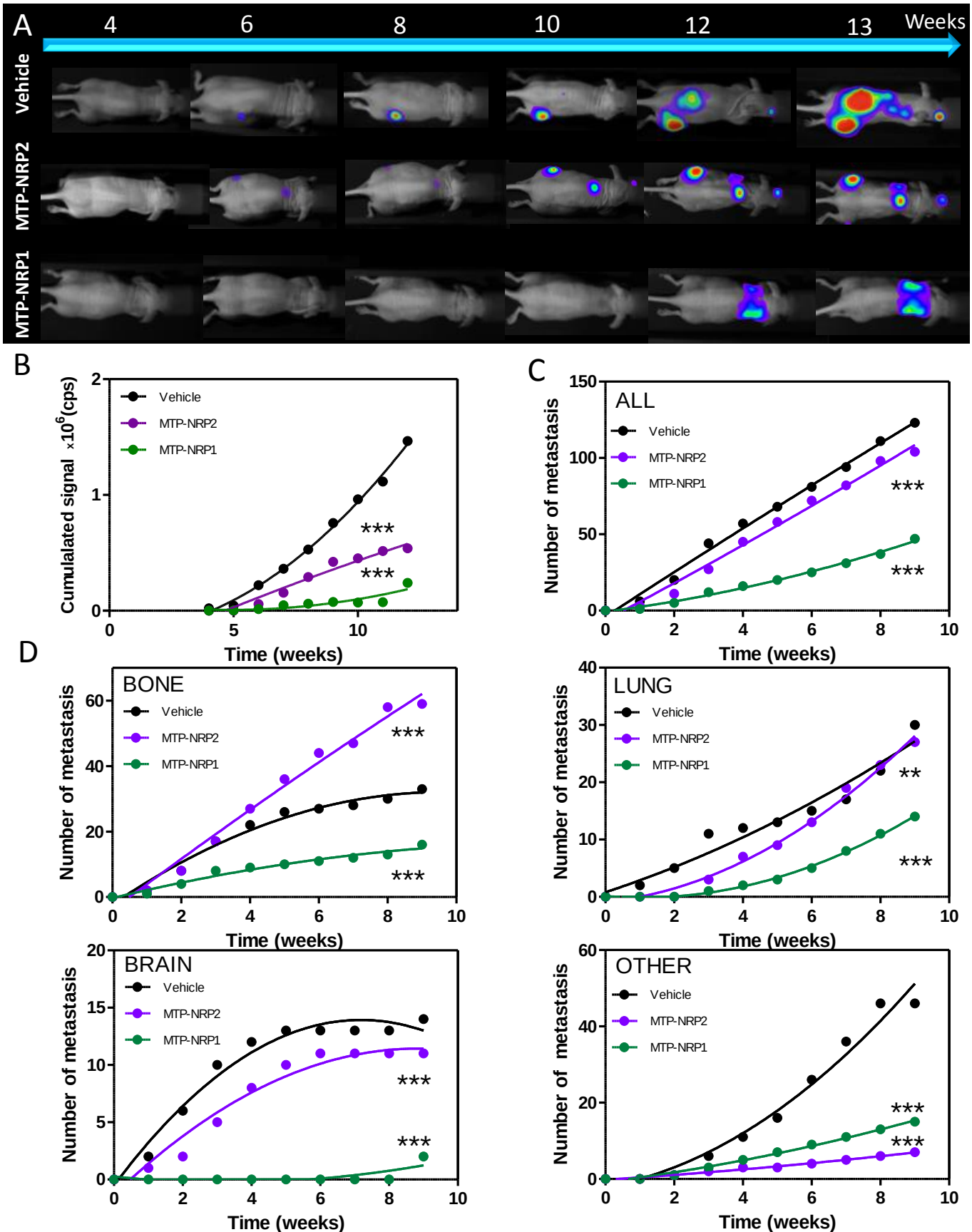


Figure 5: MTP-NRP1 improves overall survival but not MTP-NRP2

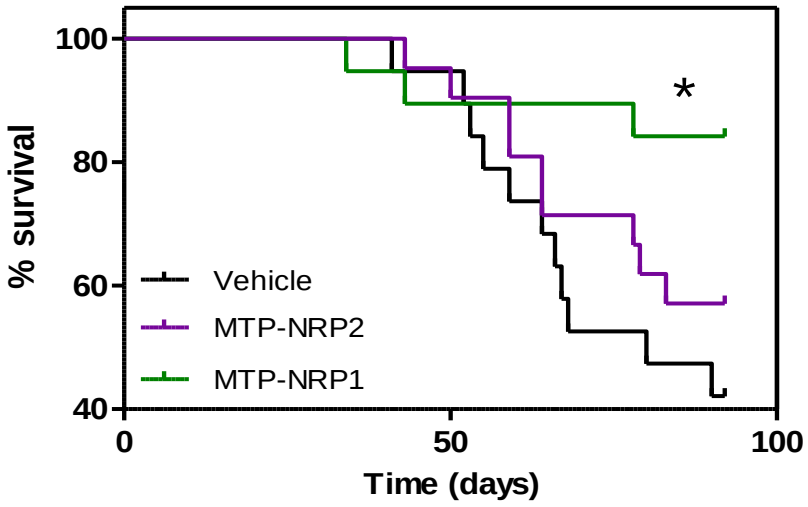


Table 1: Biochemistry analysis of blood samples of animals under MTP-NRP1 treatment

ANOVA/Bonferroni's Test	Mean Diff.	T	P < 0.05?	Summary
LDH cont vs LDH NRP1	261.7	3.308	Yes	**
ASAT cont vs ASAT NRP1	140.5	2.649	No	ns
ALAT cont vs ALAT NRP1	-5.859	0.1105	No	ns
ALP cont vs ALP NRP1	2.222	0.03997	No	ns
Creat cont vs Creat NRP1	-0.8238	0.01611	No	ns
Albumin cont vs Albumin NRP1	1.368	0.02674	No	ns
Bilirubin cont vs Bilirubin NRP1	-0.7233	0.01188	No	ns

Table 2: Numeration analysis of blood samples of animals under MTP-NRP1 treatment

ANOVA/Bonferroni's Test	Mean Diff.	T	P < 0.05?	Summary
HGB cont vs HGB NRP1	-0.6	0.03819	No	ns
Mean Corpuscular Vol cont vs MCV NRP1	3.5	0.2228	No	ns
HCT cont vs HCT NRP1	-2.18	0.1387	No	ns
PLt cont vs PLt NRP1	-51.8	3.297	Yes	*
% Baso cont vs % Baso NRP1	0.11	0.007001	No	ns
% Large Cells cont vs % Large cells NRP1	-0.66	0.042	No	ns
% Eosino cont vs % Eosino NRP1	1.63	0.1037	No	ns
% Mono cont vs % Mono NRP1	1.31	0.08337	No	ns
% Lympho cont vs % Lympho NRP1	-31.37	1.997	No	ns

VIII) Discussion and perspectives

The main objective of my thesis was to achieve the preclinical validation of an arsenal of three peptides: MTP-NeuNT, MTP-NRP1 and MTP-NRP2 in the context of breast cancer. To this end, a similar integrated workflow was conducted for each peptide through modelling, evaluation of TMD dimerization, then to test their functional activity *in vitro* and finally to test the therapeutic potential in relevant *in vivo* breast cancer models.

VIII.1) Molecular modelling

Thanks to the extraordinary growing calculation capacities of computers, molecular modelling is becoming a very popular strategy in the process of drug design including in cancer research (Geromichalos, 2007). The wide range of molecular interactions to analyse requires multi-scale approaches ranging from atomistic molecular simulation and related modelling methods such as Brownian dynamics or ab initio molecular dynamics to modelling of protein and protein complexes (Friedman et al., 2013). Here, we used homology modelling strategy to describe the potential interaction of the NeuNT TMD peptide with the nearly full length dimeric NeuNT receptor. This has been possible using the work of Arkhipov and coworkers (Arkhipov et al., 2013) who described early this year with multiple molecular simulations the mechanism by which the TMD of EGFR is crucial for dimerization. Our work does not provide the complete scheme of the process by which the peptide disrupts the NeuNT receptor dimer but is contributing to the demonstration that molecular simulation is a relevant strategy for drug design. From the modeled interaction, one would predict that the conformational change occurring in the presence of the peptide is leading to trigger an inactive receptor. It would be very interesting to strengthen our modeling by additional simulation using coarse graining approaches giving the opportunity to challenge multiple receptors and allowing the characterization of the dimerizing interface in a dynamic way. Indeed, this is currently under investigation for Neuroplin-1 and Plexin-A1 in the framework of a collaborative project between our lab and the group of Monique Genest and Norbert Garnier (Orléans). Strikingly, the different simulations conducted up to know with for examples a PlexinA1 TMD reaching a NRP1 TMD dimer or the simulation of a NRP1 TMD with a PlexinA1 TMD dimer, revealed very complex

and dynamic processes with transitions from monomers to heterodimers or oligomers in the timeframe a few microseconds. Hence, this is exemplifying the TMD as an exquisite space for the control of receptor complex assembly. This observation is completely consistent with the classical description of the numerous interactions of NRP1 receptor with different partners in the form of dimers or oligomers. Obviously, one of the long-term goals would be to model these different interactions in silico to generate cartography of the interactions. This should be done by adding the extra or intra cellular domain for simulation to envisage how the TMD may trigger conformational changes altering receptor functions. However, the relevance of simulations nourishes from experimental data. Thus, an integrated approach combining molecular simulation with experimental research is mandatory to envisage a fruitful analysis of TMD interactions. Indeed, to address the multiplicity of the partners a two-hybrid like system was used to clarify the dimerization interfaces.

VIII.2) BACTH

Since 1996, when the initial bacterial method has been performed to evaluate the interaction of transmembrane helices in a bacterial membrane, numerous double hybrid systems have been developed to study interaction of transmembrane domains in biological membranes (such as TOXCAT, GALLEX and the BACTH). Among these, the bacterial two-hybrid system is an elegant tool compatible with the analysis of dimerization potential of our membrane targeting peptides, with either their cognate receptors, or with putative co-receptor existing in the signaling platform of interest. Here, the analysis of the interaction between TMD is done in a lipid bilayer providing a relevant hydrophobic cell membrane, even if the double membrane of Escherichia coli bacteria exhibits different constraints that could probably bias the analysis. The BACTH method exhibits another limitation because proteins are fused to the T25 and T18 subunit of the CyA potentially impeding protein folding and stability thereby compromising potential interaction with partner proteins. Moreover, the output of this assay is the activation of the lactose operon by cAMP and this process involves a complex signalling cascade which could be affected by unrelated other signals (Reznikoff, 1992). Besides, this system is conducted with exogenous plasmids translated by bacteria having a post-translational machinery different from eukaryotic cells that could alter proteins interactions. It has been reported that for small

membrane proteins such as TMD unspecific week signals may be observed (Battesti and Bouveret, 2012) this is the reason why controls with unrelated protein such as GPA have been achieved in parallel. TMD of interest are expressed from two plasmid harbouring distinct replication origins giving rise to an asymmetric system, exhibiting different copy numbers. To circumvent this issue, results were presented as the mean values obtained with both plasmids. Our results could be further consolidated in a second similar assay using the GALLEX system. In contrast to the BACTH system, GALLEX also evaluates the heterodimerization potential of membrane proteins in the Escherichia coli membrane, but measures the repression of a reporter gene. The stronger the transmembrane helix dimerizes, the lesser the reporter gene is activated (Schneider and Engelman, 2003). Here the activity of the reporter gene is directly related to the formation of LexA dimers without the prior production of cAMP, leading to a direct readout. LexA is a transcription factor composed of an N-terminal DNA binding domain and a C-terminal dimerization domain. The repression of the reporter gene relies here on the binding potential of two specific LexA DNA binding domains on the reporter promoter. In contrast to BACTH, this binding site requirement leads to congestion at the juxtamembrane space reducing accessibility for other interaction also limited to the same space. For this reason, the BACTH system may provide a better quantitative signal thanks to the high solubility of cAMP. As our collaborator in Marseille generated a collection of more than 150 plasmidic constructions encoding the TMD of bitopic receptors compatible with the BACTH system, the evaluation of new TMD dimerizing partners was achievable (Sawma et al., 2013, under revision). This raised the possibility to discover new targets and therefore to develop a battery of novel therapeutic peptides. Using this collection, homodimers and heterodimers of NRP1 and NRP2 TMD interaction have already been proved (Sawma et al., 2013, under revision). Moreover in the present study, it was also possible to monitor the interactions between all the ErbB members, this across different species (human, mice and rat). While not being fully quantitative due to the limitations previously exposed, this system revealed positive and negative interactions extended to other receptors. Indeed MTP-NeuNT binds all mouse ErbB TMDs and human HER2 TMD but not with other human ErbBs nor VEGFR TMDs family members (sharing 100% homology between human and murine species), suggesting that our peptide impacts

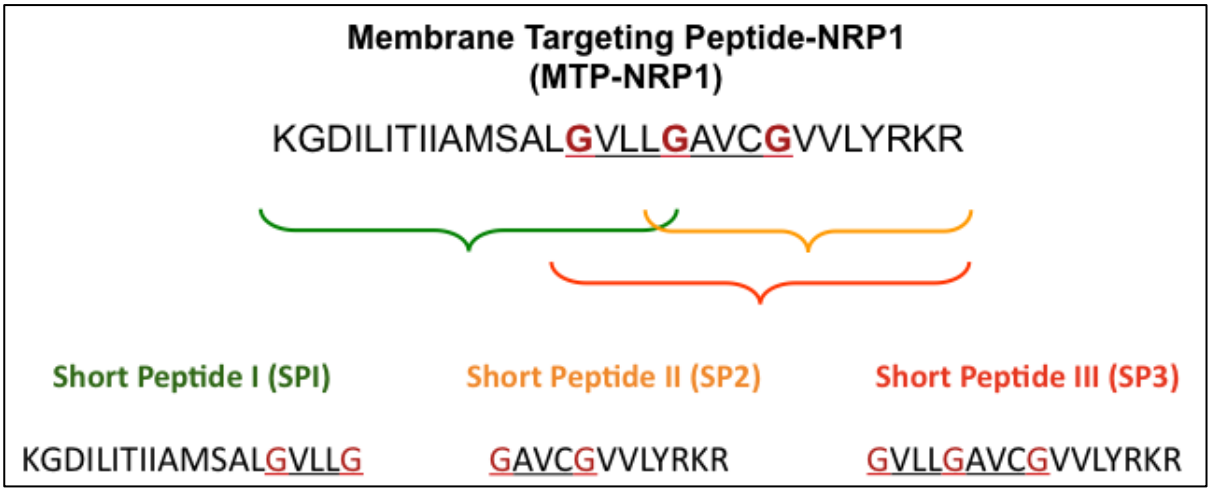


Figure 26: Determining of the functional minimum motif using short peptides

specifically the signaling platform of the Neu receptor. Next steps will be to use the BACTH system with mutant variant of the NeuNT TMD in order to identify optimized versions of the interacting peptide (to identify stronger interaction with the target or presenting extended interacting capacity with co-receptors). Hence, this would allow us to better define the minimal motif required to block the NeuNT receptor.

VIII.3) Dimerizing interface

The GxxxG motif has major implication in various pathological situations (Bargmann and Weinberg, 1988; Orian-Rousseau and Ponta, 2008). This motif has been described as highly important in the receptor dimerization for all ErbB members (Mendrola et al., 2002) and for NRP1 (Roth et al., 2008). A continuing effort in the lab is to further dissect the exact motif leading the driving force of dimerization. In this context, short peptides comprising one or the other GxxxG motif (figure 26) have already been synthesized to target NRP1 receptor. Each exhibited different functions upon *in vitro* and *in vivo* analysis. This raised the possibility that one motif or one region of the TMD may be specifically dedicated to a given function of the receptor (proliferation, migration, differentiation…). To answer this question further investigations have to be conducted and this is currently undergoing with the collaboration of IPSEN pharmaceutical company highly interested in the therapeutic potential of the short peptides. Importantly, none of these short peptides showed clear improvement of efficacy when compared with the full-length peptide. Hence, this may be an indirect demonstration that the full length is the optimal sequence for maximal efficacy. However, synthesis and purification of shorter versions of TM peptides would certainly be easier. Indeed, when determining the GRAVY index (Grand average of hydropathy index indicating the solubility of proteins)(Kyte and Doolittle, 1982), Short peptide 2 (SP2) exhibited a reduced hydrophobic index (0.525) compared to the one of the full-length peptide (1.730). The two other short peptides exhibit however similar GRAVY index (SP1=1.726; SP3=1.106). This may confer to SP2 a better biodistribution profile. Working with shorter peptides may also reduce the risk of immunogenicity. However, results obtained in the lab showed that neither full-length nor short versions of the peptides induced immune reaction in the mice. Thus, shorter versions may only conserve economic advantages with better compatibility with large batch production for use in human clinic. The

question of optimization remains open in the case of MTP-NeuNT because this peptide showed very modest if any interacting capacities with HER2 co-receptors. We could use BACTH and/or modelling to identify a sequence allowing the simultaneous inhibition of all of the HER family. From our results, this may be a chimeric peptide mixing mouse and human sequences. Further links between computational data, double hybrid investigation assays and TMD function assessment through *in vitro*/*in vivo* studies are needed to fully use the TMD as a new terra for drug design.

VIII.4) Peptides functionality

According to Hanahan and Weinberg in 2011, in the process of tumorigenesis, malignancy acquired by a cell comprises: sustained proliferative signaling, evasion from growth suppression, avoidance of immune destruction, enabled replicative immortality, tumor-promoted inflammation, activated invasion and metastasis, induced angiogenesis, genome instability and mutation, resistance to cell death, and deregulation of cellular metabolism (Hanahan and Weinberg, 2011). The ideal anti-cancer drug should impede all these hallmarks of malignant cells. Such a dreamed drug is obviously not feasible because acting at very different levels in the cell. However, we chose our targets because they are implicated at least in many of these hallmarks: proliferation, migration, metastasis, angiogenesis and cell death.

VIII.4.1) Impact on proliferation

As described in details in the introduction, breast cancers can be classified as a function of ER, PR and HER2 receptor expressions giving rise to eight phenotypes harbouring considerable differences in survival and tumor characteristics (Onitilo et al., 2009; Parise et al., 2009). Therefore, *in vitro* proliferation assays were conducted on multiple breast cancer cell lines. When analysing literature for the presence of the receptors of interest (NRP1, NRP2 and Neu/HER2) conflicting conclusion were spotted. To address this issue, IHC and QPCR analyses were assessed on all the cell lines to validate the presence of the targeted receptors prior any *in vitro* or *in vivo* assays. As for the receptors of interest, the validation of the presence or absence of estrogen and progesterone receptors was also assessed using QPCR analysis. Thus we validated different cell lines for our analysis of cell proliferation conducted with MTT assays.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is a tretrazolium salt bearing a yellow color in solution and is taken up by living cells and reduced to purple formazan in the mitochondria. This is therefore reflecting cell proliferation but also cell death. This method however doesn't allow studying proliferation overtime in a continuing manner and no information on the cell cycle can be extracted. Alternative methods using impedancemetry, such as the xCELLigence technology (Ke et al., 2011), currently developed in the lab, could be a decent alternative because allowing live monitoring of cell growth. Combined to classical cell cycle analysis using flow cytometry, we then would be able to dissect the exact timing of peptide efficacy. This may be important because upon Herceptin treatment, SKBR3 cells exhibited significant decrease in the percentage of cells in the S phase (Mayfield et al., 2001) while knocking down NRP1 in U373 cells, using specific siRNA provoked an accumulation of cells in the G1 phase (Huang, 2011). Therefore FACS analysis should be achieved to further dissect the impact of peptides on cell cycle. But in contrast to FACS analysis, the MTT method allows quick, straightforward and very simple evaluation of drugs on cell proliferation compatible with screening. Moreover this interesting method allowed us to investigate both the acute toxicity of a compound, when running the assay within 4h, as well as the impact of a chronic treatment on cell proliferation, when done after 48h or even 72h depending of the cell line. MTP-NeuNT peptide indeed revealed an acute toxicity when tested on 4T1 cells for 4h, and similar results were obtained with MTP-NRP1 and MTP-NRP2 on other breast cancer cell lines.

Importantly, the analysis of cell proliferation was conducted using cell lines not expressing the target receptors. Indeed, when adding MTP-NeuNT on MDA-MB-231 cells (triple negative) no impact on proliferation was observed. In contrast MTP-NeuNT significantly inhibited the proliferation of ErbB2-expressing cells (MCF7, SKBR3 and 4T1 cell). Hence, there were so far, very few descriptions of NRP1 or NRP2 as regulator of cell proliferation. Our data show that the inhibition of the two receptors using MTP-NRP1 or MTP-NRP2 peptides efficiently blocked proliferation of MDA-MB -231 cells. This is extremely important in the context of breast cancer because triple negative patients suffer from the lack of efficient treatment for this particular cancer type (André and Zielinski, 2012).

Assay	Strengths	Limits
Corneal pocket	•cornea avascular and transparent (all vessels seen are new vessels) •monitor progression of angiogenesis •good bioavailability of the drug if administrated in the pocket •transgenic host possible	•surgical procedure demanding •space for material limited •inflammatory process •site relevance? •biodisponibility of compound if systemic injection •expensive •time consuming
CAM	•whole animal assay stimulation possible (VEGF) •ethical •large scale screening • good bioavailability of the drug •multiple test sites possible	•host (chicken cells) compatible with drug? •inexpensive •well vascularized •undergo rapid morphological change masking the new capillaries forming (CAM developmental angiogenesis occurs within the CAM up to 11 days) •inflammation •membrane sensitive to changes in oxygen tension
Matrigel plug	•ease to administer •avascular plug • good bioavailability of the drug if injected in the plug •transgenic host possible	•matrigel costly •variation in protein composition
Retinal angiogenesis	•fast histological procedure •transgenic host possible •well defined sequence of events •parallel analysis of vessels maturation, tip cells, artery/vein tropism achievable	• complex bioavailability of compounds •retina dissection •age of different litters

Table 13: Strengths and limits of some in vivo angiogenesis assays (Auerbach et al., 1991; Tahergorabi and Khazaei, 2012)

VIII.4.2) Impact on angiogenesis

As previously mentioned, tumor angiogenesis is a critical phase of cancer progression. Consistently, several therapeutic strategies intend to block tumor-induced new blood vessels formation. Thus, the evaluation of the anti-angiogenic potential of cancer drug is important. Indeed, the tube like formation assay offers an interesting model for first evaluation of drugs. In this assay cells are grown on a 3D matrix made of Matrigel, a complex mixture of proteins secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells. This substrate is creating an exquisite pro-angiogenic extracellular matrix factors leading endothelial cells, in our case HUVEC cells, to form tube-like structures mimicking the beginning of an angiogenic process. While rapid and compatible with a screening approach, this model should only be seen as a first intention test because angiogenesis requires more than cell alignment and adherence. Thus, it is important to validate results *in vivo*. There are different options available such as the corneal pocket assay, the Matrigel plug assay, the vascularization of the chick chorio-allantoic membrane or the retinal angiogenesis assay (Auerbach et al., 2003). All of them have advantages and drawbacks that can be summarized in table 13. I decided here to use the *in vivo* mouse retinal angiogenesis assay that was successfully developed in the lab by Laurent Jacob. This allowed me to confirm the anti-angiogenic effect of MTP-NRP1 while identifying a mild but significant effect of MTP-NRP2. The amplitude of anti-angiogenic effects may look very small. In fact, when used in this model, Bevacizumab (Avastin) induced 22% reduction at 50mg/kg, a concentration 3000 fold higher than the one of MTP peptides (Benedito et al., 2012). Thus, our peptides exhibit really significant anti-angiogenic effects. It would be now interesting to repeat these experiments when increasing the concentration of the two peptides as a readout of the maximal efficient dose. The biodistribution profile of MTP-NRP2 has not been tested so far. Thus we cannot exclude that the reduced efficacy relates to problems of solubility and diffusion. However, the two peptides have 80% homology and MTP-NRP2 is less hydrophobic than MTP-NRP1 with a GRAVY index of 1.436. It is important to mention that improved angiogenesis and expression of VEGF was linked with overexpression of HER2 in human tumor cells. Moreover, Herceptin has been shown to inhibit VEGF expression (Kumar and Yarmand-Bagheri, 2001). Hence, the anti-angiogenic activity of MTP-ErbB2 should be searched. Preliminary data I

TG mouse model	Tumor incidence (%)	Tumor latency (months)	Metastasis incidence (%)	Metastatic site	Metastasis latency (months)	Mutation site	Reference
MMTV-Neu WT	100	6.8	72	Lung	8	-	Guy et al., 1992
MMTV-Neu NT	100	3 - 5	20	Lung	3.5	TMD Val_{664} to Glu_{664}	Muller et al., 1988; Bouchard et al., 1989
MMTV-Neu YB	100	6	65	Lung	2	TKD Tyr_{1144} to Phe_{1144}	Dankort et al., 2001
MMTV-Neu YD	100	3.6	44	Lung	2	TKD Tyr_{1227} to Phe_{1227}	Dankort et al., 2001
MMTV-Neu YPD	35	7	20	Lung	1 - 2	TKD no phosphorylation sites left	Dankort et al., 2001
MMTV-Neu NDL	80	5.4	52	Lung	1 - 2	ECD 12 (NDL1) and 5 (NDL2) aa deletion	Siegel et al., 1999
NeuNT knockin	45	12	Not reported	Not reported	Not reported	TMD Val_{664} to Glu_{664}	Andrechek et al., 2000
Dox inducible NeuNT	100	1.5	92	Lung	Not reported	TMD Val_{664} to Glu_{664}	Moody et al., 2002

Table 14: MMTV-Neu related transgenic mouse models of breast cancer adapted and completed from Fantozzi and Christofori (2006), TMD: transmembrane domain, TKD: tyrosin kinase domain, NeuTPD: Neu Tyrosin Phosphorylation Deficient, Neu NDL: Neu DeLetion mutant.

obtained with the tube-like structure assay actually showed a significant anti-angiogenic effect of the peptide (-49% compared to control conditions). I also started to collect samples of culture medium to measure VEGF production of cancer cells grown with or without peptides to evaluate the impact on the autocrine VEGF-induced cell proliferation and survival. This should then lead us to *in vivo* assays or direct monitoring of blood vessels contents in tumor samples using classical immunohistological analysis using blood vessel markers such as CD31 or Isolectin-B4.

VIII.5) Modelling breast cancer and its metastases: One size does not fit all peptides

VIII.5.1) The transgenic mouse model MMTV-NeuNT accurately sizes for MTP-NeuNT

Up to date more than 50 transgenic mouse models of breast cancer have been generated (Taneja et al., 2009b) and roughly half harbor the MMTV promoter (Fantozzi and Christofori, 2006). Various MMTV-Neu related transgenic breast cancer mouse model exist and each offers its own characteristics in term of tumor latency, incidence and lung metastasis occurrence. The table 14 summarizes the most common MMTV-Neu related transgenic breast cancer mouse model.

The driving force that led us to choose the MMTV-NeuNT transgenic mice rather than another was based on multiple criteria: first, to evaluate the peptidic strategy targeting ErbB2 on primary breast cancer and metastases, second, the relevance to human cancer, and third the time frame. To answer the first criteria, of course besides Neu driven breast cancer mice models numerous other models can lead to breast cancer (at least 50 different models are available) such as the PyMT mouse model (Lin et al., 2003) or the MMTV-cyclinD1 mouse model (Wang et al., 1994). Willing to target the ErbB2 receptor, the Neu driven trangenic models was obvious. In MMTV-NeuNT, the breast tumorigenesis is driven by a point mutation within the transmembrane domain on the Neu receptor, region of particular interest in my thesis work. Moreover, this point mutation is occurring in a dimerization motif. Therefore, all transgenic mice exhibiting a mutation in the tyrosine kinase domain (Dankort et al., 2001) were not of

great interest, such as MMTV-NeuNT YB, YD, YPD. In the same way mutation in the extracellular domain, as observed in the MMTV-Neu NDL transgenic mice are less interesting than mutations occurring in the TMD. Concerning the relevance to human cancer, HER2 amplification occurs in 20 to 30% of human mammary carcinoma, therefore Neu receptor is of particular interest as it is the murine counterpart receptor. MMTV-NeuNT transgenic mice model mimics the ductal carcinoma in situ (DCIS) observed in humans and more precisely the comedocarcinomas type (Fantozzi and Christofori, 2006) knowing that 80% of in situ human breast cancers are DCIS. Additionally, cells are able to escape from primary tumor to form metastases to the lungs, the second most common site of metastasis in human. Importantly, human breast tumors arise spontaneously; therefore the doxycycline inducible NeuNT transgenic mice was not relevant neither, also because tumors regress with the loss of ErbB2 expression upon the withdrawal of doxycycline. For NeuNT knock-in transgenic mice no metastases are reported probably due to the very long latency before observation of the first primary tumor. Most importantly, MMTV-NeuNT transgenic mouse model has been clearly shown to be highly predictive of drug efficacy in human (Roberts et al., 2012). Regarding the time frame criteria, NeuNT knock-in transgenic mice carry the activated Neu oncogene under the transcriptional control of the endogenous Neu promoter specifically in the mammary epithelium. Interestingly, tumorigenesis in this model was associated with selective amplification of the activated Neu allele (2–22 copies) correlating with elevated levels of Neu transcript and protein. Here mice also developed comedo-adenocarcinomas such as in the MMTV-NeuNT transgenic model. However, focal mammary tumors arise in these mice after a very long latency period (over 12months). Moreover, the penetrance in mice is very low (45%) and no metastases are reported rendering this model not as efficient as the MMTV-NeuNT model. Hence, the integration of all of these criteria identified the MMTV-NeuNT transgenic mouse model (in the FVB background due to a higher susceptibility to develop mammary tumors compared to C57/Bl6 mice) as the best compromise to test whether our peptidic strategy would impede breast tumor growth and metastasis progression. We had the same discussion around the development of MTP-NRP1 and MTP-NRP2 therapeutic peptides because a transgenic mouse model developing tumors exhibiting triple negative-like phenotype exists. These mice harbor tumors following BRAC1

deficiency, however, these mice exhibit again a very long tumor latency (above 7months), they harbor reduced penetrance of the phenotype (less than 20% in some models), and poor lung metastasis occurrence. Moreover mice may need ionizing radiation to produce tumors, a criterion less relevant to human disease (Jones et al., 2005; Diaz-Cruz et al., 2010). For these reasons we decided to use tumor cell grafting paradigms.

VIII.5.2) The triple negative breast cancer cell-grafted mice model completely sizes for MTP-NRP1 and MTP-NRP2

VIII.5.2.1) Orthotopic breast cancer model

Bridging the gap between mouse and human is always a challenging task in the field of drug development. The production of mice with deficient immune system allowed the emergence of xenograft models consisting in grafting human cells under the skin of nude mice. However, this approach turned out to be poorly predictive because only a limited number of therapeutic agents tested in mice harboured a significant clinical efficacy at well-tolerated doses in human (Bibby, 2004). According to Corbett and co-workers most of the agents that had entered the clinic in the 80s presented low or no activity against most of the xenografted tumors in mice (Corbett et al., 1987). In contrast to xenograft models that do not take into account the microenvironment normally encountered by tumor cells, orthotopic grafting (brain tumor cells grated into the brain or breast cancer cells grafted into the mammary fat pad for examples) reproduces the primary site of human cancer to better mimic the clinical situation. Hence, as it reflects the biological features enabling cancer growth, this model is considered to provide better prediction of the potential clinical activity for a given drug. Moreover, thanks to the use of genetically modified cancer cell lines expressing fluorescent or luminescent reporter genes, it is now possible to achieve longitudinal studies allowing precise monitoring of tumor growth. Here, I performed orthotopic grafting of MDA-MB-231 expressing luciferase. Strikingly, while forming large and easily detectable tumors, we never observed metastasis at the time point of sacrifice. In fact, the tumor mass is developing so quick that mice were eventually dying or reaching ethical limit points before the end of the protocol compromising the chance to reach the critical size of metastasis to be detectable with bioluminescence (few micrometres in diameter). Thus, to assess the anti-metastatic property of MTP-NRP1 and MTP-NRP2 in this

orthotopic model, one possibility would be to surgically remove the primary tumor and maintain treatments until metastasis detection. This may probably give a chance to observe metastases issued from the primary site as already been observed (Elliott et al., 1992; Fu et al., 1993).

In spite of some variability of the size of the tumors (particularly in the control groups), this method turns out to be efficient and allowed me to prove the anti-tumor effect of MTP-NRP1 and MTP-NRP2. However, the evaluation of the anti-metastatic effect had to be performed using an alternative model.

VIII.5.2.2) Intracardiac injection

Metastasis formation remains the most significant problem in the field of breast cancer. The intracardiac injection model provides several advantages for metastasis investigation. Importantly, the tropism of MDA-MB-231 for certain organs closely mimics the human metastasis anatomic location throughout the body: that is bone, lungs, brain and liver. This is not achieved using other sites of injection. Indeed, tail vein injection leads to lung metastasis mainly while intra-splenic or portal vein injections lead to liver metastasis (basically cell seed in the first capillary bed encountered from the injection site). Another important advantage is that the time course for metastasis detection is relatively short (1 month) in comparison with metastasis occurring from primary tumor (at least 3 months).

The main disadvantage of this model is that early process of metastasis is skipped. Indeed metastatic cells are assumed to leave the primary tumor site, migrate and intravasate into blood or lymphatic vessels. Then cells are able to transit in these vessels, resisting to shear stress and anoikis (programmed cell death associated with loss of cellular contact), and to finally attach to vessel wall to extravasate in the distant organ (Sahai, 2007). This final step is the only one achieved when performing intracardiac injection. Moreover, cells escaping from the initial tumor are very specific cells that had acquired evading and migrating capabilities. Therefore injecting a heterogeneous population of cells abolishes the selection events occurring in the primary tumor site that yield a distinct profile of successful metastasis. In contrast to the orthotopic mouse model, intracardiac injection is technically more challenging and some mice don’t survive the procedure.

Upon intracardiac injection, using the same luciferases cells as in the orthotopic mouse model, I was able to achieved longitudinal monitoring of metastasis number and extension and site of onset. Using this technique I was able to prove the anti-metastatic property of MTP-NRP1 peptide and this was observed in all sites analysed. Even though MTP-NRP2 harboured a significant reduction both in overall metastasis extent and overall number, when analysing the impact on bone metastasis number, a clear increase was spotted. This analysis is of major importance knowing that the primary site of metastasis in human pathology is bone (Solomayer et al., 2000; Berman et al., 2013). Interestingly, neuropilin-2 expression has been linked with a physiological role in bone homeostasis. Indeed, very recently, immunohistochemistry analysis demonstrated positive NRP2 signal in both osteoblastic and osteoclastic cells of the long bones in mice (Verlinden et al., 2013). As exposed in the introduction, when metastasizing to bone, cancer cells will modify the local microenvironment by recruiting and modulating the behavior of the host cells, notably activating osteoclast. Noteworthy, osteoclasts are responsible of bone modeling while osteoblasts increase bone mass. According to Verlinden and collaborators, NRP2 knockout mice exhibited reduced bone mass suggesting a decrease in osteoblast activity, along with an improved number of osteoclasts and a reduced osteoblast count. The addition of NRP2 inhibiting peptide may therefore lead to the inhibition of osteoblast, favoring bone reduction further strengthened by metastatic cells colonizing bone. It would be interesting to generate NRP2- deficient MDA-MB-231 cells to verify whether the observed increase of bone metastases is the consequence of the treatment (inhibition of osteoblast) or the modification of tumor cell phenotype rendering them more aggressive for bone.

It is doubtful that the complexity of cancer in human patients can be completely modelled by a unique animal experimental system. Indeed there is no ideal mouse model to answer a specific biological question.

VIII.6) Towards clinics?

Developing peptides targeting transmembrane domains is a very innovative mechanism of action to inhibit membrane-spanning proteins implicated in cancer. Up to now no drugs based on this strategy are to my knowledge being developed in cancer biology. The pioneering

peptidic strategy harbours already numerous advantages over conventional drugs, and numerous studies have been assessed to place the peptides on the path towards clinics.

VIII.6.1) Optimal solubilisation condition

A key issue in drug development is the ability to obtain a drug with formulation compatible with clinical use. In our case, peptide solubilisation is achieved by dilution in a 72μM lithium-dodecyl-sulphate (LDS) solution following the suggestions of Penin and coworkers (personal communication) who successfully used this detergent to solubilize membrane proteins. Interestingly detergents, such as LDS, are small amphipatic molecules exhibiting the ability to protect proteins, such as our peptides, from aqueous media. Upon solubilisation LDS concentration is ten times above the critical micellar concentration (CMC) allowing the incorporation of the peptides within micelles. Importantly during the solubilisation process, trifluoro-ethanol (in acetic acid) is used in order to favour the alpha helical structure of the peptide. This structure is mandatory for the peptide function and is obviously maintained when peptides are in solution. This has been verified using circular dichroism showing up to 95% alpha helical structure of peptides upon solubilisation with LDS. When adding the peptide to cells or when administrating the compound *in vivo*, as very low dosages are used to reach the peptide efficacy, the LDS concentration drops below the CMC favouring then integration of the peptides in the cell membrane.However, little is known about the toxicity and elimination of LDS that is not yet used as a standard solvent of hydrophobic drugs in the pharmaceutical industry. This is not the case of DMSO (dimethyl sulfoxide) widely used during initial drug discovery (Stegemann et al., 2007). This may represent an alternative solvent that could better fulfil regulatory constraints in the process of clinical development. Indeed, several experiments in the lab showed that peptides dissolved in DMSO exhibit similar biological properties. Hence, one last alternative would be the use of amphipols (Popot et al., 2011, 2003). These amphiphatic polymers exhibit a central hydrophobic cage and external hydrophilic chains and were deigned to stabilize hydrophobic protein such as our peptides in aqueous condition. Here again, *in vivo* assay conducted in the lab showed that amphipol-embedded peptides (MTP-NRP1) had equivalent activity as LDS solubilized peptides in a model of subcutaneous xenograft of brain

tumors in mice. Such flexibility for the choice of solvent is undoubtedly an asset for future clinical development of the MTP drugs.

VIII.6.2) What makes TMD peptides good drug candidates?

VIII.6.2.1) TMD peptides are first in class drugs

A good knowledge of the action mechanism of a new drug is mandatory in the process of drug development. My work contributes to the accumulation of evidence over the last 10 years leading to the description of the first class of receptor complex association interfering drugs. The combination of FRET assay, biochemical assay, immunoprecipitation assays, signalling assays, now associated with molecular simulations, highlight the existence of a very specific interacting capacity of TMD peptides for which we need now to establish and formalize an interactome-like cartography. Hence, upon interaction with its target, a given TMD peptide is able to alter the formation and or stability of the corresponding signalling platform thereby disrupting related biological functions.

VIII.6.2.2) TMD peptides exhibit long term efficacy

One of the striking features of our TMD peptide strategy is the long lasting effect observed both *in vitro* and *in vivo*. As described in the result sections, MTP-NeuNT exhibited for example a significant effect up to 72h, an effect intensified at 48h. This is consistent with our previous description of the cellular trafficking of fluorescent versions of TM peptides that can reach *in vitro* cell membrane within 45-60 minutes and can be detected there up to 96h before endocytosis and lysosomal degradation (Bennasroune et al., 2004; Roth et al., 2008). This persistent activity is counterintuitive because peptidic drugs usually exhibit short half-life time rarely exceeding few minutes *in vivo*. The particular behaviour of TMD peptides is probably due the insertion in the lipidic bilayer protecting the peptides from most of proteases. Only intra-membrane cleaving protease (I-CliPs) may exhibit the property to cleave membrane proteins (Erez et al., 2009) and therefore our membrane targeting peptides. Besides this indirect property, work in the lab is currently exploring how the TMD peptides may perturb receptor recycling. In brief, receptors such as NRP1 undergo continuing recycling cycles following endocytic pathways and co-internalisation with signalling receptors. One mechanism of action

would be the inhibition of this process, leading to accumulation of the target and the inhibitory peptide at the cell surface.

VIII.6.2.3) TMD peptides are non-immunogenic

As previously mentioned peptides would rapidly reach cell membrane and stay there *in vivo*. This is also hiding the antigenic sites of the peptides rendering it not accessible to the immune system. Also, the hydrophobicity of a protein is known to compromise immune surveillance (Naim and van Oss, 1992). A company specialized in the production of antibody by the mean of coupling the antigen to the KLH protein carrier (Biotem) failed to produce antibody against NRP1 TMD in mice. This is demonstrating that immunogenicity, that is often a major risk in clinical drug development, may not be an issue in our case.

VIII.6.2.4) TMD peptides act at very low dosage

According to Herceptin patent (http://www.google.com/patents/EP1106183A2?cl=en&hl=fr, EP 1106183 A2), muMab 4D5 (the most potent mouse HER2 antibody used in preclinical drug development) was used in xenograft tumor models at a concentration of 10mg/kg. Concerning NRP1, the function-blocking antibody developed by Genentech exhibit anti-tumor activity with dosages ranging from 5 to 10mg/kg (Liang et al., 2007; Pan et al., 2007). Starzec and coworkers in 2006 also identified a peptide inhibiting VEGF binding to NRP1 and reducing tumor volume in an orthotopic mammary carcinoma model of MDA-MB-231 cells at a dosage of 20mg/kg. Hence, our studies demonstrate that TMD peptides exhibit their inhibitory role with concentration 1000-20 000 inferior to standard drugs.

VIII.6.2.5) Faster towards clinics

Importantly in the preclinical-drug development process, teratogenicity and genotoxicity studies are assessed. Noteworthy, it is not expected that peptides would interact directly with DNA or other chromosomal material. Therefore these time-consuming and cost-consuming studies are not needed in peptide derived pharmaceuticals drugs (Guidance for industry, S6 Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals, FDA regulatory information guidance, 1997 http://www.fda.gov/downloads/regulatoryinformation/guidances/ucm129171.pdf.)

VIII.6.2.7) TMD peptides exhibit unexpected good biodistribution

Previous work achieved in the lab demonstrated that the IP injection of a rhodaminated peptide (Rhod-MTP-NRP1) could largely diffuse in the entire body and in the tumor of subcutaneous xenografted-mice (Nasarre et al., 2010). Moreover, when analysing tissue and tumor sections, it was shown that peptides can exit from the blood circulation and penetrate in the surrounding tissue. Similar results were also obtained with biotinylated versions of the NRP1 peptide. It would be useful to perform a systematic analysis of the biodistribution profiles of fluorescent versions of all of the different therapeutic peptides we had developed. However, the method used so far is poorly quantitative and remains essentially qualitative. This is the reason why to further validate the biodistribution profile of the compound we intended to perform SPECT imaging. This approach requires the use of radio-labelled peptides. This work was done in a collaborative framework with the IMABIO group in IPHC. Unfortunately, due to the high hydrophobic nature of the peptide, iodinated peptides could not be achieved yet due to purification issues. The recent progress made by Polypeptide, the company in charge of producing a GMP batch of NRP1-peptide for regulatory clinical development may open the possibility to solve this issue. Hence, we should be able to perform soon detailed PK/PD analysis of our therapeutic peptides.

VIII.6.3) What's next?

The work I accomplished during my PhD definitively validates the proof of concept of the proposed therapeutic strategy. Concerning MTP-NeuNT, we obviously will design another peptide to target human HER2 receptor. Our preliminary *in vitro* data suggest promising results on HER2 expressing cells (SKBR3 and MCF7). In the human context, it will be interesting to compare and/or combine MTP-HER2 with Herceptin. Indeed, 30% to 50% of HER2 over expressing breast cancer are primary resistant to Trastuzumab. This is the consequence of several mechanisms altering the HER2-trastuzumab complex as exposed in the introduction: a) the antibody binding site can be masked or block by cleavage of ErbB2 ECD or overexpression of MUC4 (Mucin-4 a membrane ErbB2 ligand); b) ErbB2 downstream signaling can be altered through down regulation of PTEN tumor suppressor gene and PI3K mutant activation enhancing ErbB2 signaling network; c) inhibition of cell cycle growth arrest by the loss of cyclin-dependent

kinase inhibitor p27; d) compensation mechanism by signaling through other ErbB receptors or non-ErbB receptors such as Insulin growth factor receptor (IGFR) (Fang et al., 2011). Additionally, the most common side effect of Trastuzumab is cardiotoxicity (Wicki et al., 2012). The use of MTP-HER2 could overpass some of the resistance mechanism describe above as the peptide is insensitive to ligand and is supposed to destabilize the whole signalling platform. It has to be checked whether the low concentration used would reduce the non-specific cardiac toxicity. Hence, targeting MTP-NRP1 and MTP-NRP2 in the breast context appears to be a very promising strategy and the combination of these peptides with MTP-ErbB should be performed.

IX) CONCLUSION

The use of membrane targeting peptides definitively represents a unique, original new strategy in preclinical drug development. Even if the flowchart of each peptides was not yet fully identical, and more analysis have to be conducted for one or the other peptide, combining in a ordered manner molecular modelling, the double hybrid system, *in vitro* and *in vivo* analysis offers an outstanding integrative approach for the evaluation of the therapeutic potential of the strategy. I was able to demonstrated that the peptidic strategy was applicable to breast cancer, tackling with a widely validated receptor HER2, and to prove that NRP1 is an interesting new target in metastatic breast context. Concerning NRP2 receptor, further analysis need to be achieve in order to better understand the role of this receptor in breast cancer particularly at the level of bone metastasis formation. Noteworthy, the need of new classes of drug is of highly importance in metastatic breast cancers and especially in triple negative breast cancers exhibiting the worse outcome. Additional studies should evaluate the combination of both MTP-NRP1 and MTP-ErbB2 peptides in the breast context as my result exposed for both very promising clinical outcomes. Over a decade, numerous studies achieved in our team, supplemented with my data, offer convincing body of evidence supporting the interest to bring these drugs towards the patient's bed. From terra incognita to the exploration of a world of hope, the heart of the membrane is undoubtedly becoming a new promising estate for drug design.

Bibliography

Akagi, M., M. Kawaguchi, W. Liu, M.F. McCarty, A. Takeda, F. Fan, O. Stoeltzing, A.A. Parikh, Y.D. Jung, C.D. Bucana, P.F. Mansfield, D.J. Hicklin, and L.M. Ellis. 2003. Induction of neuropilin-1 and vascular endothelial growth factor by epidermal growth factor in human gastric cancer cells. *Br. J. Cancer*. 88:796–802. doi:10.1038/sj.bjc.6600811.

Allred, D.C., and D. Medina. 2008. The Relevance of Mouse Models to Understanding the Development and Progression of Human Breast Cancer. *J. Mammary Gland Biol. Neoplasia*. 13:279–288. doi:10.1007/s10911-008-9093-5.

Alroy, I., and Y. Yarden. 1999. Biochemistry of HER2 Oncogenesis in Breast Cancer. *Breast Dis.* 11:31–48.

André, F., and C.C. Zielinski. 2012. Optimal strategies for the treatment of metastatic triple-negative breast cancer with currently approved agents. *Ann. Oncol.* 23:vi46–vi51. doi:10.1093/annonc/mds195.

Andrechek, E.R., W.R. Hardy, A.A. Girgis-Gabardo, R.L.S. Perry, R. Butler, F.L. Graham, R.C. Kahn, M.A. Rudnicki, and W.J. Muller. 2002. ErbB2 is required for muscle spindle and myoblast cell survival. *Mol. Cell. Biol.* 22:4714–4722.

Arkhipov, A., Y. Shan, R. Das, N.F. Endres, M.P. Eastwood, D.E. Wemmer, J. Kuriyan, and D.E. Shaw. 2013. Architecture and membrane interactions of the EGF receptor. *Cell*. 152:557–569. doi:10.1016/j.cell.2012.12.030.

Auerbach, R., R. Lewis, B. Shinners, L. Kubai, and N. Akhtar. 2003. Angiogenesis assays: a critical overview. *Clin. Chem.* 49:32–40.

Bachelder, R.E., A. Crago, J. Chung, M.A. Wendt, L.M. Shaw, G. Robinson, and A.M. Mercurio. 2001. Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. *Cancer Res.* 61:5736–5740.

Bachelder, R.E., E.A. Lipscomb, X. Lin, M.A. Wendt, N.H. Chadborn, B.J. Eickholt, and A.M. Mercurio. 2003. Competing autocrine pathways involving alternative neuropilin-1 ligands regulate chemotaxis of carcinoma cells. *Cancer Res.* 63:5230–5233.

Bachelder, R.E., M.A. Wendt, and A.M. Mercurio. 2002. Vascular endothelial growth factor promotes breast carcinoma invasion in an autocrine manner by regulating the chemokine receptor CXCR4. *Cancer Res.* 62:7203–7206.

Bagnard, D., M. Lohrum, D. Uziel, A.W. Püschel, and J. Bolz. 1998. Semaphorins act as attractive and repulsive guidance signals during the development of cortical projections. *Dev. Camb. Engl.* 125:5043–5053.

Bagri, A., and M. Tessier-Lavigne. 2002a. Neuropilins as Semaphorin receptors: in vivo functions in neuronal cell migration and axon guidance. *Adv. Exp. Med. Biol.* 515:13–31.

Bagri, A., and M. Tessier-Lavigne. 2002b. Neuropilins as Semaphorin receptors: in vivo functions in neuronal cell migration and axon guidance. *Adv. Exp. Med. Biol.* 515:13–31.

Bagri, A., M. Tessier-Lavigne, and R.J. Watts. 2009. Neuropilins in tumor biology. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 15:1860–1864. doi:10.1158/1078-0432.CCR-08-0563.

Bargmann, C.I., M.C. Hung, and R.A. Weinberg. 1986. Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185. *Cell*. 45:649–657.

Bargmann, C.I., and R.A. Weinberg. 1988. Increased tyrosine kinase activity associated with the protein encoded by the activated neu oncogene. *Proc. Natl. Acad. Sci. U. S. A.* 85:5394–5398.

Battesti, A., and E. Bouveret. 2012. The bacterial two-hybrid system based on adenylate cyclase reconstitution in Escherichia coli. *Methods San Diego Calif*. 58:325–334. doi:10.1016/j.ymeth.2012.07.018.

Bazley, L.A., and W.J. Gullick. 2005. The epidermal growth factor receptor family. *Endocr. Relat. Cancer*. 12 Suppl 1:S17–27. doi:10.1677/erc.1.01032.

Benedito, R., S.F. Rocha, M. Woeste, M. Zamykal, F. Radtke, O. Casanovas, A. Duarte, B. Pytowski, and R.H. Adams. 2012. Notch-dependent VEGFR3 upregulation allows angiogenesis without VEGF-VEGFR2 signalling. *Nature*. 484:110–114. doi:10.1038/nature10908.

Bennasroune, A., M. Fickova, A. Gardin, S. Dirrig-Grosch, D. Aunis, G. Crémel, and P. Hubert. 2004. Transmembrane peptides as inhibitors of ErbB receptor signaling. *Mol. Biol. Cell*. 15:3464–3474. doi:10.1091/mbc.E03-10-0753.

Bennasroune, A., A. Gardin, C. Auzan, E. Clauser, S. Dirrig-Grosch, M. Meira, A. Appert-Collin, D. Aunis, G. Crémel, and P. Hubert. 2005. Inhibition by transmembrane peptides of chimeric insulin receptors. *Cell. Mol. Life Sci. CMLS*. 62:2124–2131. doi:10.1007/s00018-005-5226-9.

Berman, A.T., A.D. Thukral, W.-T. Hwang, L.J. Solin, and N. Vapiwala. 2013. Incidence and patterns of distant metastases for patients with early-stage breast cancer after breast conservation treatment. *Clin. Breast Cancer*. 13:88–94. doi:10.1016/j.clbc.2012.11.001.

Bibby, M.C. 2004. Orthotopic models of cancer for preclinical drug evaluation: advantages and disadvantages. *Eur. J. Cancer*. 40:852–857. doi:10.1016/j.ejca.2003.11.021.

Bielenberg, D.R., C.A. Pettaway, S. Takashima, and M. Klagsbrun. 2006. Neuropilins in neoplasms: expression, regulation, and function. *Exp. Cell Res.* 312:584–593. doi:10.1016/j.yexcr.2005.11.024.

Bloom, H.J.G., and W.W. Richardson. 1957. Histological Grading and Prognosis in Breast Cancer. *Br. J. Cancer*. 11:359–377.

Bocharov, E.V., P.E. Volynsky, K.V. Pavlov, R.G. Efremov, and A.S. Arseniev. 2010. Structure elucidation of dimeric transmembrane domains of bitopic proteins. *Cell Adhes. Migr.* 4:284–298.

Børresen-Dale, A.-L. 2003. TP53 and breast cancer. *Hum. Mutat.* 21:292–300. doi:10.1002/humu.10174.

Brenton, J.D., L.A. Carey, A.A. Ahmed, and C. Caldas. 2005. Molecular Classification and Molecular Forecasting of Breast Cancer: Ready for Clinical Application? *J. Clin. Oncol.* 23:7350–7360. doi:10.1200/JCO.2005.03.3845.

Brisken, C., and B. O'Malley. 2010. Hormone action in the mammary gland. *Cold Spring Harb. Perspect. Biol.* 2:a003178. doi:10.1101/cshperspect.a003178.

Brooks, B.R., R.E. Bruccoleri, B.D. Olafson, D.J. States, S. Swaminathan, and M. Karplus. 1983. CHARMM: A program for macromolecular energy, minimization, and dynamics calculations. *J. Comput. Chem.* 4:187–217. doi:10.1002/jcc.540040211.

Cackowski, F.C., L. Xu, B. Hu, and S.-Y. Cheng. 2004. Identification of two novel alternatively spliced Neuropilin-1 isoforms. *Genomics*. 84:82–94. doi:10.1016/j.ygeno.2004.02.001.

Callahan, R., U. Mudunuri, S. Bargo, A. Raafat, D. McCurdy, C. Boulanger, W. Lowther, R. Stephens, B.T. Luke, C. Stewart, X. Wu, D. Munroe, and G.H. Smith. 2012. Genes affected by mouse mammary tumor virus (MMTV) proviral insertions in mouse mammary tumors are deregulated or mutated in primary human mammary tumors. *Oncotarget*. 3:1320–1334.

Carraway, K.L., V.P. Ramsauer, B. Haq, and C.A. Carothers Carraway. 2003. Cell signaling through membrane mucins. *BioEssays News Rev. Mol. Cell. Dev. Biol.* 25:66–71. doi:10.1002/bies.10201.

Casazza, A., V. Finisguerra, L. Capparuccia, A. Camperi, J.M. Swiercz, S. Rizzolio, C. Rolny, C. Christensen, A. Bertotti, I. Sarotto, M. Risio, L. Trusolino, J. Weitz, M. Schneider, M. Mazzone, P.M. Comoglio, and L. Tamagnone. 2010. Sema3E-Plexin D1 signaling drives human cancer cell invasiveness and metastatic spreading in mice. *J. Clin. Invest.* 120:2684–2698. doi:10.1172/JCI42118.

Case, D.A., T.E. Cheatham, T. Darden, H. Gohlke, R. Luo, K.M. Merz, A. Onufriev, C. Simmerling, B. Wang, and R.J. Woods. 2005. The Amber biomolecular simulation programs. *J. Comput. Chem.* 26:1668–1688. doi:10.1002/jcc.20290.

Castro-Rivera, E., S. Ran, P. Thorpe, and J.D. Minna. 2004. Semaphorin 3B (SEMA3B) induces apoptosis in lung and breast cancer, whereas VEGF165 antagonizes this effect. *Proc. Natl. Acad. Sci. U. S. A.* 101:11432–11437. doi:10.1073/pnas.0403969101.

Caunt, M., J. Mak, W.-C. Liang, S. Stawicki, Q. Pan, R.K. Tong, J. Kowalski, C. Ho, H.B. Reslan, J. Ross, L. Berry, I. Kasman, C. Zlot, Z. Cheng, J. Le Couter, E.H. Filvaroff, G. Plowman, F. Peale, D. French, R. Carano, A.W. Koch, Y. Wu, R.J. Watts, M. Tessier-Lavigne, and A. Bagri. 2008. Blocking neuropilin-2 function inhibits tumor cell metastasis. *Cancer Cell*. 13:331–342. doi:10.1016/j.ccr.2008.01.029.

Chambers, A.F., A.C. Groom, and I.C. MacDonald. 2002. Dissemination and growth of cancer cells in metastatic sites. *Nat. Rev. Cancer*. 2:563–572. doi:10.1038/nrc865.

Chasis, J.A., and N. Mohandas. 1992. Red blood cell glycophorins. *Blood*. 80:1869–1879.

Cheatham, B., S.E. Shoelson, K. Yamada, E. Goncalves, and C.R. Kahn. 1993. Substitution of the erbB-2 oncoprotein transmembrane domain activates the insulin receptor and modulates the action of insulin and insulin-receptor substrate 1. *Proc. Natl. Acad. Sci. U. S. A.* 90:7336–7340.

Chen, H., A. Chédotal, Z. He, C.S. Goodman, and M. Tessier-Lavigne. 1997. Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. *Neuron*. 19:547–559.

Cheng, H.J., A. Bagri, A. Yaron, E. Stein, S.J. Pleasure, and M. Tessier-Lavigne. 2001. Plexin-A3 mediates semaphorin signaling and regulates the development of hippocampal axonal projections. *Neuron*. 32:249–263.

Chothia, C., M. Levitt, and D. Richardson. 1981. Helix to helix packing in proteins. *J. Mol. Biol.* 145:215–250.

Chotteau-Lelievre, A., R. Montesano, J. Soriano, P. Soulie, X. Desbiens, and Y. de Launoit. 2003. PEA3 transcription factors are expressed in tissues undergoing branching morphogenesis and promote formation of duct-like structures by mammary epithelial cells in vitro. *Dev. Biol.* 259:241–257.

Christensen, C., N. Ambartsumian, G. Gilestro, B. Thomsen, P. Comoglio, L. Tamagnone, P. Guldberg, and E. Lukanidin. 2005. Proteolytic processing converts the repelling signal Sema3E into an inducer of invasive growth and lung metastasis. *Cancer Res.* 65:6167–6177. doi:10.1158/0008-5472.CAN-04-4309.

Chu, D., and J. Lu. 2008a. Novel therapies in breast cancer: what is new from ASCO 2008. *J. Hematol. Oncol.J Hematol Oncol*. 1:16. doi:10.1186/1756-8722-1-16.

Chu, D., and J. Lu. 2008b. Novel therapies in breast cancer: what is new from ASCO 2008. *J. Hematol. Oncol.J Hematol Oncol*. 1:16. doi:10.1186/1756-8722-1-16.

Chumsri, S., T. Howes, T. Bao, G. Sabnis, and A. Brodie. 2011. Aromatase, aromatase inhibitors, and breast cancer. *J. Steroid Biochem. Mol. Biol.* 125:13–22. doi:10.1016/j.jsbmb.2011.02.001.

Clegg, R.M. 1995. Fluorescence resonance energy transfer. *Curr. Opin. Biotechnol.* 6:103–110.

Corbett, T.H., F.A. Valeriote, and L.H. Baker. 1987. Is the P388 murine tumor no longer adequate as a drug discovery model? *Invest. New Drugs*. 5:3–20.

Cowin, P., and J. Wysolmerski. 2010. Molecular Mechanisms Guiding Embryonic Mammary Gland Development. *Cold Spring Harb. Perspect. Biol.* 2. doi:10.1101/cshperspect.a003251.

Cymer, F., and D. Schneider. 2010. Transmembrane helix-helix interactions involved in ErbB receptor signaling. *Cell Adhes. Migr.* 4:299–312.

Dankort, D., B. Maslikowski, N. Warner, N. Kanno, H. Kim, Z. Wang, M.F. Moran, R.G. Oshima, R.D. Cardiff, and W.J. Muller. 2001. Grb2 and Shc Adapter Proteins Play Distinct Roles in Neu (ErbB-2)-Induced Mammary Tumorigenesis: Implications for Human Breast Cancer. *Mol. Cell. Biol.* 21:1540–1551. doi:10.1128/MCB.21.5.1540-1551.2001.

Dawson, J.P., J.S. Weinger, and D.M. Engelman. 2002. Motifs of serine and threonine can drive association of transmembrane helices. *J. Mol. Biol.* 316:799–805. doi:10.1006/jmbi.2001.5353.

Demchenko, A.P. 2010. Advanced Fluorescence Reporters in Chemistry and Biology I: Fundamentals and Molecular Design. Springer. 393 pp.

Diaz-Cruz, E.S., M.C. Cabrera, R. Nakles, B.H. Rutstein, and P.A. Furth. 2010. BRCA1 deficient mouse models to study pathogenesis and therapy of triple negative breast cancer. *Breast Dis.* 32:85–97. doi:10.3233/BD-2010-0308.

Duffy, M.J., E. McKiernan, N. O'Donovan, and P.M. McGowan. 2009. The role of ADAMs in disease pathophysiology. *Clin. Chim. Acta Int. J. Clin. Chem.* 403:31–36.

Dumont, D.J., L. Jussila, J. Taipale, A. Lymboussaki, T. Mustonen, K. Pajusola, M. Breitman, and K. Alitalo. 1998. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science*. 282:946–949.

Durrant, J.D., and J.A. McCammon. 2011. Molecular dynamics simulations and drug discovery. *BMC Biol.* 9:71. doi:10.1186/1741-7007-9-71.

Eccles, S.A. 2011a. The epidermal growth factor receptor/Erb-B/HER family in normal and malignant breast biology. *Int. J. Dev. Biol.* 55:685–696. doi:10.1387/ijdb.113396se.

Eccles, S.A. 2011b. The epidermal growth factor receptor/Erb-B/HER family in normal and malignant breast biology. *Int. J. Dev. Biol.* 55:685–696. doi:10.1387/ijdb.113396se.

Eisenhauer, E.A., P. Therasse, J. Bogaerts, L.H. Schwartz, D. Sargent, R. Ford, J. Dancey, S. Arbuck, S. Gwyther, M. Mooney, L. Rubinstein, L. Shankar, L. Dodd, R. Kaplan, D. Lacombe, and J. Verweij. 2009. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur. J. Cancer Oxf. Engl. 1990*. 45:228–247. doi:10.1016/j.ejca.2008.10.026.

Elliott, B.E., S.P. Tam, D. Dexter, and Z.Q. Chen. 1992. Capacity of adipose tissue to promote growth and metastasis of a murine mammary carcinoma: effect of estrogen and progesterone. *Int. J. Cancer J. Int. Cancer*. 51:416–424.

Ellis, L.M. 2006. The role of neuropilins in cancer. *Mol. Cancer Ther.* 5:1099–1107. doi:10.1158/1535-7163.MCT-05-0538.

Endocrine Treatments for Breast Cancer. *Aboutcom Breast Cancer*.

Erez, E., D. Fass, and E. Bibi. 2009. How intramembrane proteases bury hydrolytic reactions in the membrane. *Nature*. 459:371–378. doi:10.1038/nature08146.

Erickson, S.L., K.S. O'Shea, N. Ghaboosi, L. Loverro, G. Frantz, M. Bauer, L.H. Lu, and M.W. Moore. 1997. ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2-and heregulin-deficient mice. *Dev. Camb. Engl.* 124:4999–5011.

Eroles, P., A. Bosch, J. Alejandro Pérez-Fidalgo, and A. Lluch. 2012. Molecular biology in breast cancer: Intrinsic subtypes and signaling pathways. *Cancer Treat. Rev.* 38:698–707. doi:10.1016/j.ctrv.2011.11.005.

Fang, L., Z. Barekati, B. Zhang, Z. Liu, and X. Zhong. 2011a. Targeted therapy in breast cancer: what's new? *Swiss Med. Wkly.* 141:w13231. doi:10.4414/smw.2011.13231.

Fang, L., Z. Barekati, B. Zhang, Z. Liu, and X. Zhong. 2011b. Targeted therapy in breast cancer: what's new? *Swiss Med. Wkly.* doi:10.4414/smw.2011.13231.

Fantozzi, A., and G. Christofori. 2006. Mouse models of breast cancer metastasis. *Breast Cancer Res. BCR*. 8:212. doi:10.1186/bcr1530.

Fatunmbi, M., J. Shelton, and S.M. Aronica. 2012. MMP-9 increases HER2/neu expression and alters apoptosis levels in human mammary epithelial cells (HMEC). *Breast Cancer Res. Treat.* 135:519–530. doi:10.1007/s10549-012-2191-5.

Ferguson, K.M., P.J. Darling, M.J. Mohan, T.L. Macatee, and M.A. Lemmon. 2000. Extracellular domains drive homo- but not hetero-dimerization of erbB receptors. *EMBO J.* 19:4632–4643. doi:10.1093/emboj/19.17.4632.

Fernandis, A.Z., A. Prasad, H. Band, R. Klösel, and R.K. Ganju. 2004. Regulation of CXCR4-mediated chemotaxis and chemoinvasion of breast cancer cells. *Oncogene*. 23:157–167. doi:10.1038/sj.onc.1206910.

Fisher, L.E., D.M. Engelman, and J.N. Sturgis. 1999. Detergents modulate dimerization, but not helicity, of the glycophorin A transmembrane domain. *J. Mol. Biol.* 293:639–651. doi:10.1006/jmbi.1999.3126.

Fleishman, S.J., J. Schlessinger, and N. Ben-Tal. 2002. A putative molecular-activation switch in the transmembrane domain of erbB2. *Proc. Natl. Acad. Sci.* 99:15937–15940. doi:10.1073/pnas.252640799.

Fong, G.H., J. Rossant, M. Gertsenstein, and M.L. Breitman. 1995. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature*. 376:66–70. doi:10.1038/376066a0.

Freudenberg, J.A., Q. Wang, M. Katsumata, J. Drebin, I. Nagatomo, and M.I. Greene. 2009. The role of HER2 in early breast cancer metastasis and the origins of resistance to HER2-targeted therapies. *Exp. Mol. Pathol.* 87:1–11. doi:10.1016/j.yexmp.2009.05.001.

Friedman, R., K. Boye, and K. Flatmark. 2013. Molecular modelling and simulations in cancer research. *Biochim. Biophys. Acta*. 1836:1–14. doi:10.1016/j.bbcan.2013.02.001.

Fu, X., P. Le, and R.M. Hoffman. 1993. A metastatic orthotopic-transplant nude-mouse model of human patient breast cancer. *Anticancer Res.* 13:901–904.

Fujita, H., B. Zhang, K. Sato, J. Tanaka, and M. Sakanaka. 2001. Expressions of neuropilin-1, neuropilin-2 and semaphorin 3A mRNA in the rat brain after middle cerebral artery occlusion. *Brain Res.* 914:1–14.

Gabrovska, P.N., R.A. Smith, T. Tiang, S.R. Weinstein, L.M. Haupt, and L.R. Griffiths. 2011. Semaphorin-plexin signalling genes associated with human breast tumourigenesis. *Gene*. 489:63–69. doi:10.1016/j.gene.2011.08.024.

Gagnon, M.L., D.R. Bielenberg, Z. Gechtman, H.Q. Miao, S. Takashima, S. Soker, and M. Klagsbrun. 2000. Identification of a natural soluble neuropilin-1 that binds vascular endothelial growth factor: In vivo expression and antitumor activity. *Proc. Natl. Acad. Sci. U. S. A.* 97:2573–2578. doi:10.1073/pnas.040337597.

Gassmann, M., F. Casagranda, D. Orioli, H. Simon, C. Lai, R. Klein, and G. Lemke. 1995. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature*. 378:390–394. doi:10.1038/378390a0.

George, S.R., S.P. Lee, G. Varghese, P.R. Zeman, P. Seeman, G.Y. Ng, and B.F. O'Dowd. 1998. A transmembrane domain-derived peptide inhibits D1 dopamine receptor function without affecting receptor oligomerization. *J. Biol. Chem.* 273:30244–30248.

Gerber, D., N. Sal-Man, and Y. Shai. 2004. Two motifs within a transmembrane domain, one for homodimerization and the other for heterodimerization. *J. Biol. Chem.* 279:21177–21182. doi:10.1074/jbc.M400847200.

Geromichalos, G.D. 2007. Importance of molecular computer modeling in anticancer drug development. *J. BUON Off. J. Balk. Union Oncol.* 12 Suppl 1:S101–118.

Ghosh, S., C.A.W. Sullivan, M.P. Zerkowski, A.M. Molinaro, D.L. Rimm, R.L. Camp, and G.G. Chung. 2008. High levels of vascular endothelial growth factor and its receptors (VEGFR-1, VEGFR-2, neuropilin-1) are associated with worse outcome in breast cancer. *Hum. Pathol.* 39:1835–1843. doi:10.1016/j.humpath.2008.06.004.

Giraudo, E., L. Primo, E. Audero, H.P. Gerber, P. Koolwijk, S. Soker, M. Klagsbrun, N. Ferrara, and F. Bussolino. 1998. Tumor necrosis factor-alpha regulates expression of vascular endothelial growth factor receptor-2 and of its co-receptor neuropilin-1 in human vascular endothelial cells. *J. Biol. Chem.* 273:22128–22135.

Goel, H.L., D. Bae, B. Pursell, L.M. Gouvin, S. Lu, and A.M. Mercurio. 2011. Neuropilin-2 promotes branching morphogenesis in the mouse mammary gland. *Dev. Camb. Engl.* 138:2969–2976. doi:10.1242/dev.051318.

Goldgar, D.E., S. Healey, J.G. Dowty, L.D. Silva, X. Chen, A.B. Spurdle, M.B. Terry, M.J. Daly, S.M. Buys, M.C. Southey, I. Andrulis, E.M. John, $author firstName $author.lastName, $author firstName $author.lastName, K.K. Khanna, J.L. Hopper, P.J. Oefner, S. Lakhani, and G. Chenevix-Trench. 2011. Rare variants in the ATM gene and risk of breast cancer. *Breast Cancer Res.* 13:R73. doi:10.1186/bcr2919.

Göllner, G.P., G. Müller, R. Alt, J. Knop, and A.H. Enk. 2000. Therapeutic application of T cell receptor mimic peptides or cDNA in the treatment of T cell-mediated skin diseases. *Gene Ther.* 7:1000–1004. doi:10.1038/sj.gt.3301183.

Grandclement, C., J.R. Pallandre, S. Valmary Degano, E. Viel, A. Bouard, J. Balland, J.-P. Rémy-Martin, B. Simon, A. Rouleau, W. Boireau, M. Klagsbrun, C. Ferrand, and C. Borg. 2011a. Neuropilin-2 expression promotes TGF-β1-mediated epithelial to mesenchymal transition in colorectal cancer cells. *PloS One*. 6:e20444. doi:10.1371/journal.pone.0020444.

Grandclement, C., J.R. Pallandre, S. Valmary Degano, E. Viel, A. Bouard, J. Balland, J.-P. Rémy-Martin, B. Simon, A. Rouleau, W. Boireau, M. Klagsbrun, C. Ferrand, and C. Borg. 2011b. Neuropilin-2 expression promotes TGF-β1-mediated epithelial to mesenchymal transition in colorectal cancer cells. *PloS One*. 6:e20444. doi:10.1371/journal.pone.0020444.

Grewal, J., and S. Kesari. 2008. Breast cancer surface receptors predict risk for developing brain metastasis and subsequent prognosis. *Breast Cancer Res. BCR*. 10:104. doi:10.1186/bcr1868.

Gu, C., and E. Giraudo. 2013. The role of semaphorins and their receptors in vascular development and cancer. *Exp. Cell Res.* 319:1306–1316. doi:10.1016/j.yexcr.2013.02.003.

Guy, C.T., M.A. Webster, M. Schaller, T.J. Parsons, R.D. Cardiff, and W.J. Muller. 1992. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc. Natl. Acad. Sci. U. S. A.* 89:10578–10582.

Haiman, C.A., D.O. Stram, I. Cheng, E.E. Giorgi, L. Pooler, K. Penney, L.L. Marchand, B.E. Henderson, and M.L. Freedman. 2006. Common Genetic Variation at PTEN and Risk of Sporadic Breast and Prostate Cancer. *Cancer Epidemiol. Biomarkers Prev.* 15:1021–1025. doi:10.1158/1055-9965.EPI-05-0896.

Hanahan, D., and R.A. Weinberg. 2011. Hallmarks of cancer: the next generation. *Cell*. 144:646–674. doi:10.1016/j.cell.2011.02.013.

Hardy, K.M., B.W. Booth, M.J.C. Hendrix, D.S. Salomon, and L. Strizzi. 2010. ErbB/EGF Signaling and EMT in Mammary Development and Breast Cancer. *J. Mammary Gland Biol. Neoplasia*. 15:191–199. doi:10.1007/s10911-010-9172-2.

He, L., N. Shobnam, and K. Hristova. 2011. Specific inhibition of a pathogenic receptor tyrosine kinase by its transmembrane domain. *Biochim. Biophys. Acta*. 1808:253–259. doi:10.1016/j.bbamem.2010.08.007.

He, Z., and M. Tessier-Lavigne. 1997. Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. *Cell*. 90:739–751.

Hebert, T.E., S. Moffett, J.P. Morello, T.P. Loisel, D.G. Bichet, C. Barret, and M. Bouvier. 1996. A peptide derived from a beta2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. *J. Biol. Chem.* 271:16384–16392.

Hernandez-Aya, L.F., and A.M. Gonzalez-Angulo. 2011. Targeting the Phosphatidylinositol 3-Kinase Signaling Pathway in Breast Cancer. *The Oncologist*. 16:404–414. doi:10.1634/theoncologist.2010-0402.

Holbro, T., and N.E. Hynes. 2004. ErbB receptors: directing key signaling networks throughout life. *Annu. Rev. Pharmacol. Toxicol.* 44:195–217. doi:10.1146/annurev.pharmtox.44.101802.121440.

Hong, Y.-K., N. Harvey, Y.-H. Noh, V. Schacht, S. Hirakawa, M. Detmar, and G. Oliver. 2002. Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. *Dev. Dyn. Off. Publ. Am. Assoc. Anat.* 225:351–357. doi:10.1002/dvdy.10163.

Huang, Y. 2011. RNA interference targeting NRP-1 inhibits human glioma cell proliferation and enhances cell apoptosis. *Mol. Med. Reports*. doi:10.3892/mmr.2011.550.

Hubert, P., P. Sawma, J.-P. Duneau, J. Khao, J. Hénin, D. Bagnard, and J. Sturgis. 2010. Single-spanning transmembrane domains in cell growth and cell-cell interactions: More than meets the eye? *Cell Adhes. Migr.* 4:313–324. doi:10.4161/cam.4.2.12430.

Hudis, C.A. 2007. Trastuzumab--mechanism of action and use in clinical practice. *N. Engl. J. Med.* 357:39–51. doi:10.1056/NEJMra043186.

Hussein, O., and S.V. Komarova. 2011. Breast cancer at bone metastatic sites: recent discoveries and treatment targets. *J. Cell Commun. Signal.* 5:85–99. doi:10.1007/s12079-011-0117-3.

Huynh, N.T., R.A. Ffrench, R.A. Boadle, and N. Manolios. 2003. Transmembrane T-cell receptor peptides inhibit B- and natural killer-cell function. *Immunology*. 108:458–464. doi:10.1046/j.1365-2567.2003.01614.x.

Hynes, N.E., and C.J. Watson. 2010. Mammary gland growth factors: roles in normal development and in cancer. *Cold Spring Harb. Perspect. Biol.* 2:a003186. doi:10.1101/cshperspect.a003186.

Jackson, C., D. Browell, H. Gautrey, and A. Tyson-Capper. 2013. Clinical Significance of HER-2 Splice Variants in Breast Cancer Progression and Drug Resistance. *Int. J. Cell Biol.* 2013:973584. doi:10.1155/2013/973584.

Jocic, Z., and R.D. Staton. 1993. Reduplication after right middle cerebral artery infarction. *Brain Cogn.* 23:222–230. doi:10.1006/brcg.1993.1056.

Johnson, R.M., A. Rath, and C.M. Deber. 2006. The position of the Gly-xxx-Gly motif in transmembrane segments modulates dimer affinity. *Biochem. Cell Biol. Biochim. Biol. Cell.* 84:1006–1012. doi:10.1139/o06-192.

Jones, L.P., M. Li, E.D. Halama, Y. Ma, R. Lubet, C.J. Grubbs, C.-X. Deng, E.M. Rosen, and P.A. Furth. 2005. Promotion of mammary cancer development by tamoxifen in a mouse model of Brca1-mutation-related breast cancer. *Oncogene*. 24:3554–3562. doi:10.1038/sj.onc.1208426.

Jubb, A.M., L.A. Strickland, S.D. Liu, J. Mak, M. Schmidt, and H. Koeppen. 2012. Neuropilin-1 expression in cancer and development. *J. Pathol.* 226:50–60. doi:10.1002/path.2989.

Karimova, G., A. Ullmann, and D. Ladant. 2001. Protein-protein interaction between Bacillus stearothermophilus tyrosyl-tRNA synthetase subdomains revealed by a bacterial two-hybrid system. *J. Mol. Microbiol. Biotechnol.* 3:73–82.

Kaur, H., S. Mao, S. Shah, D.H. Gorski, S.A. Krawetz, B.F. Sloane, and R.R. Mattingly. 2013. Next-generation sequencing: a powerful tool for the discovery of molecular markers in breast ductal carcinoma in situ. *Expert Rev. Mol. Diagn.* 13:151–165. doi:10.1586/erm.13.4.

Kawakami, A., T. Kitsukawa, S. Takagi, and H. Fujisawa. 1996. Developmentally regulated expression of a cell surface protein, neuropilin, in the mouse nervous system. *J. Neurobiol.* 29:1–17. doi:10.1002/(SICI)1097-4695(199601)29:1<1::AID-NEU1>3.0.CO;2-F.

Kawasaki, T., T. Kitsukawa, Y. Bekku, Y. Matsuda, M. Sanbo, T. Yagi, and H. Fujisawa. 1999. A requirement for neuropilin-1 in embryonic vessel formation. *Dev. Camb. Engl.* 126:4895–4902.

Ke, N., X. Wang, X. Xu, and Y.A. Abassi. 2011. The xCELLigence system for real-time and label-free monitoring of cell viability. *Methods Mol. Biol. Clifton NJ*. 740:33–43. doi:10.1007/978-1-61779-108-6_6.

Konecny, G.E., Y.G. Meng, M. Untch, H.-J. Wang, I. Bauerfeind, M. Epstein, P. Stieber, J.-M. Vernes, J. Gutierrez, K. Hong, M. Beryt, H. Hepp, D.J. Slamon, and M.D. Pegram. 2004. Association between HER-2/neu and vascular endothelial growth factor expression predicts clinical outcome in primary breast cancer patients. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 10:1706–1716.

Kumar, R., and R. Yarmand-Bagheri. 2001. The role of HER2 in angiogenesis. *Semin. Oncol.* 28:27–32.

Kyte, J., and R.F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105–132.

Lee, T.-H., S. Seng, M. Sekine, C. Hinton, Y. Fu, H.K. Avraham, and S. Avraham. 2007. Vascular endothelial growth factor mediates intracrine survival in human breast carcinoma cells through internally expressed VEGFR1/FLT1. *PLoS Med.* 4:e186. doi:10.1371/journal.pmed.0040186.

Lemmon, M.A., J.M. Flanagan, J.F. Hunt, B.D. Adair, B.J. Bormann, C.E. Dempsey, and D.M. Engelman. 1992. Glycophorin A dimerization is driven by specific interactions between transmembrane alpha-helices. *J. Biol. Chem.* 267:7683–7689.

Li, E., W.C. Wimley, and K. Hristova. 2012. Transmembrane Helix Dimerization: Beyond the Search for Sequence Motifs. *Biochim. Biophys. Acta*. 1818:183–193. doi:10.1016/j.bbamem.2011.08.031.

Liang, W.-C., M.S. Dennis, S. Stawicki, Y. Chanthery, Q. Pan, Y. Chen, C. Eigenbrot, J. Yin, A.W. Koch, X. Wu, N. Ferrara, A. Bagri, M. Tessier-Lavigne, R.J. Watts, and Y. Wu. 2007. Function blocking antibodies to neuropilin-1 generated from a designed human synthetic antibody phage library. *J. Mol. Biol.* 366:815–829. doi:10.1016/j.jmb.2006.11.021.

Lin, E.Y., J.G. Jones, P. Li, L. Zhu, K.D. Whitney, W.J. Muller, and J.W. Pollard. 2003. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. *Am. J. Pathol.* 163:2113–2126. doi:10.1016/S0002-9440(10)63568-7.

Lofts, F.J., H.C. Hurst, M.J. Sternberg, and W.J. Gullick. 1993. Specific short transmembrane sequences can inhibit transformation by the mutant neu growth factor receptor in vitro and in vivo. *Oncogene*. 8:2813–2820.

Longo, N., R.C. Shuster, L.D. Griffin, S.D. Langley, and L.J. Elsas. 1992. Activation of insulin receptor signaling by a single amino acid substitution in the transmembrane domain. *J. Biol. Chem.* 267:12416–12419.

Lu, J., P.S. Steeg, J.E. Price, S. Krishnamurthy, S.A. Mani, J. Reuben, M. Cristofanilli, G. Dontu, L. Bidaut, V. Valero, G.N. Hortobagyi, and D. Yu. 2009. Breast cancer metastasis: challenges and opportunities. *Cancer Res.* 69:4951–4953. doi:10.1158/0008-5472.CAN-09-0099.

MacKenzie, K.R., J.H. Prestegard, and D.M. Engelman. 1997. A Transmembrane Helix Dimer: Structure and Implications. *Science*. 276:131–133. doi:10.1126/science.276.5309.131.

Magon, N. 2011. Gonadotropin releasing hormone agonists: Expanding vistas. *Indian J. Endocrinol. Metab.* 15:261–267. doi:10.4103/2230-8210.85575.

Malhotra, G.K., X. Zhao, H. Band, and V. Band. 2010. Histological, molecular and functional subtypes of breast cancers. *Cancer Biol. Ther.* 10:955–960. doi:10.4161/cbt.10.10.13879.

Manolios, N., M. Ali, and V. Bender. 2010. T-cell antigen receptor (TCR) transmembrane peptides. *Cell Adhes. Migr.* 4:273–283.

Maughan, K.L., M.A. Lutterbie, and P.S. Ham. 2010a. Treatment of breast cancer. *Am. Fam. Physician*. 81:1339–1346.

Maughan, K.L., M.A. Lutterbie, and P.S. Ham. 2010b. Treatment of breast cancer. *Am. Fam. Physician*. 81:1339–1346.

Mayfield, S., J.P. Vaughn, and T.E. Kute. 2001. DNA strand breaks and cell cycle perturbation in herceptin treated breast cancer cell lines. *Breast Cancer Res. Treat.* 70:123–129.

Melnyk, R.A., S. Kim, A.R. Curran, D.M. Engelman, J.U. Bowie, and C.M. Deber. 2004. The affinity of GXXXG motifs in transmembrane helix-helix interactions is modulated by long-range communication. *J. Biol. Chem.* 279:16591–16597. doi:10.1074/jbc.M313936200.

Mendrola, J.M., M.B. Berger, M.C. King, and M.A. Lemmon. 2002. The Single Transmembrane Domains of ErbB Receptors Self-associate in Cell Membranes. *J. Biol. Chem.* 277:4704–4712. doi:10.1074/jbc.M108681200.

Miettinen, P.J., J.E. Berger, J. Meneses, Y. Phung, R.A. Pedersen, Z. Werb, and R. Derynck. 1995. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature*. 376:337–341. doi:10.1038/376337a0.

Miloso, M., M. Mazzotti, W.C. Vass, and L. Beguinot. 1995. SHC and GRB-2 are constitutively by an epidermal growth factor receptor with a point mutation in the transmembrane domain. *J. Biol. Chem.* 270:19557–19562.

Minotti, G., P. Menna, E. Salvatorelli, G. Cairo, and L. Gianni. 2004. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol. Rev.* 56:185–229. doi:10.1124/pr.56.2.6.

Moasser, M.M. 2007a. The oncogene HER2; Its signaling and transforming functions and its role in human cancer pathogenesis. *Oncogene*. 26:6469–6487. doi:10.1038/sj.onc.1210477.

Moasser, M.M. 2007b. The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis. *Oncogene*. 26:6469–6487. doi:10.1038/sj.onc.1210477.

Moody, S.E., C.J. Sarkisian, K.T. Hahn, E.J. Gunther, S. Pickup, K.D. Dugan, N. Innocent, R.D. Cardiff, M.D. Schnall, and L.A. Chodosh. 2002. Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis. *Cancer Cell*. 2:451–461.

Moreno-Aspitia, A., and E.A. Perez. 2009. Treatment options for breast cancer resistant to anthracycline and taxane. *Mayo Clin. Proc. Mayo Clin.* 84:533–545. doi:10.1016/S0025-6196(11)60585-5.

Morris, J.K., W. Lin, C. Hauser, Y. Marchuk, D. Getman, and K.F. Lee. 1999. Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development. *Neuron*. 23:273–283.

Morris, J.S., T. Stein, M.-A. Pringle, C.R. Davies, S. Weber-Hall, R.K. Ferrier, A.K. Bell, V.J. Heath, and B.A. Gusterson. 2006. Involvement of axonal guidance proteins and their signaling partners in the developing mouse mammary gland. *J. Cell. Physiol.* 206:16–24. doi:10.1002/jcp.20427.

Muller, W.J., E. Sinn, P.K. Pattengale, R. Wallace, and P. Leder. 1988. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell*. 54:105–115.

Naim, J.O., and C.J. van Oss. 1992. The effect of hydrophilicity-hydrophobicity and solubility on the immunogenicity of some natural and synthetic polymers. *Immunol. Invest.* 21:649–662.

Nakamura, F., and Y. Goshima. 2002. Structural and functional relation of neuropilins. *Adv. Exp. Med. Biol.* 515:55–69.

Nasarre, C., M. Roth, L. Jacob, L. Roth, E. Koncina, A. Thien, G. Labourdette, P. Poulet, P. Hubert, G. Crémel, G. Roussel, D. Aunis, and D. Bagnard. 2010. Peptide-based interference of the transmembrane domain of neuropilin-1 inhibits glioma growth in vivo. *Oncogene*. 29:2381–2392. doi:10.1038/onc.2010.9.

Nasarre, P., S. Kusy, B. Constantin, V. Castellani, H.A. Drabkin, D. Bagnard, and J. Roche. 2005. Semaphorin SEMA3F has a repulsing activity on breast cancer cells and inhibits E-cadherin-mediated cell adhesion. *Neoplasia New York N*. 7:180–189. doi:10.1593/neo.04481.

Nawabi, H., A. Briançon-Marjollet, C. Clark, I. Sanyas, H. Takamatsu, T. Okuno, A. Kumanogoh, M. Bozon, K. Takeshima, Y. Yoshida, F. Moret, K. Abouzid, and V. Castellani. 2010. A midline switch of receptor processing regulates commissural axon guidance in vertebrates. *Genes Dev.* 24:396–410. doi:10.1101/gad.542510.

Negro, A., B.K. Brar, and K.-F. Lee. 2004. Essential roles of Her2/erbB2 in cardiac development and function. *Recent Prog. Horm. Res.* 59:1–12.

Neufeld, G., A.D. Sabag, N. Rabinovicz, and O. Kessler. 2012. Semaphorins in Angiogenesis and Tumor Progression. *Cold Spring Harb. Perspect. Med.* 2. doi:10.1101/cshperspect.a006718.

Nevanlinna, H., and J. Bartek. 2006. The CHEK2 gene and inherited breast cancer susceptibility. *Oncogene*. 25:5912–5919. doi:10.1038/sj.onc.1209877.

Ng, G.Y., B.F. O'Dowd, S.P. Lee, H.T. Chung, M.R. Brann, P. Seeman, and S.R. George. 1996. Dopamine D2 receptor dimers and receptor-blocking peptides. *Biochem. Biophys. Res. Commun.* 227:200–204. doi:10.1006/bbrc.1996.1489.

Nkondjock, A., and P. Ghadirian. 2005. [Risk factors and risk reduction of breast cancer]. *Médecine Sci. MS.* 21:175–180. doi:10.1051/medsci/2005212175.

Oakman, C., S. Bessi, E. Zafarana, F. Galardi, L. Biganzoli, and A. Di Leo. 2009. Recent advances in systemic therapy: new diagnostics and biological predictors of outcome in early breast cancer. *Breast Cancer Res. BCR*. 11:205. doi:10.1186/bcr2238.

Oates, J., G. King, and A.M. Dixon. 2010. Strong oligomerization behavior of PDGFbeta receptor transmembrane domain and its regulation by the juxtamembrane regions. *Biochim. Biophys. Acta*. 1798:605–615. doi:10.1016/j.bbamem.2009.12.016.

Onitilo, A.A., J.M. Engel, R.T. Greenlee, and B.N. Mukesh. 2009. Breast Cancer Subtypes Based on ER/PR and Her2 Expression: Comparison of Clinicopathologic Features and Survival. *Clin. Med. Res.* 7:4–13. doi:10.3121/cmr.2009.825.

Orian-Rousseau, V., and H. Ponta. 2008. Adhesion proteins meet receptors: a common theme? *Adv. Cancer Res.* 101:63–92. doi:10.1016/S0065-230X(08)00404-1.

Osborne, C.K., H. (Holly) Zhao, and S.A.W. Fuqua. 2000. Selective Estrogen Receptor Modulators: Structure, Function, and Clinical Use. *J. Clin. Oncol.* 18:3172–3186.

Palmieri, D., J.L. Bronder, J.M. Herring, T. Yoneda, R.J. Weil, A.M. Stark, R. Kurek, E. Vega-Valle, L. Feigenbaum, D. Halverson, A.O. Vortmeyer, S.M. Steinberg, K. Aldape, and P.S. Steeg. 2007. Her-2 Overexpression Increases the Metastatic Outgrowth of Breast Cancer Cells in the Brain. *Cancer Res.* 67:4190–4198. doi:10.1158/0008-5472.CAN-06-3316.

Pan, Q., Y. Chanthery, W.-C. Liang, S. Stawicki, J. Mak, N. Rathore, R.K. Tong, J. Kowalski, S.F. Yee, G. Pacheco, S. Ross, Z. Cheng, J. Le Couter, G. Plowman, F. Peale, A.W. Koch, Y. Wu, A. Bagri, M. Tessier-Lavigne, and R.J. Watts. 2007. Blocking Neuropilin-1 Function Has an Additive Effect with Anti-VEGF to Inhibit Tumor Growth. *Cancer Cell*. 11:53–67. doi:10.1016/j.ccr.2006.10.018.

Parikh, A.A., F. Fan, W.B. Liu, S.A. Ahmad, O. Stoeltzing, N. Reinmuth, D. Bielenberg, C.D. Bucana, M. Klagsbrun, and L.M. Ellis. 2004. Neuropilin-1 in human colon cancer: expression, regulation, and role in induction of angiogenesis. *Am. J. Pathol.* 164:2139–2151. doi:10.1016/S0002-9440(10)63772-8.

Parikh, A.A., W.B. Liu, F. Fan, O. Stoeltzing, N. Reinmuth, C.J. Bruns, C.D. Bucana, D.B. Evans, and L.M. Ellis. 2003. Expression and regulation of the novel vascular endothelial growth factor receptor neuropilin-1 by epidermal growth factor in human pancreatic carcinoma. *Cancer*. 98:720–729. doi:10.1002/cncr.11560.

Parise, C.A., K.R. Bauer, M.M. Brown, and V. Caggiano. 2009. Breast cancer subtypes as defined by the estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) among women with invasive breast cancer in California, 1999-2004. *Breast J.* 15:593–602. doi:10.1111/j.1524-4741.2009.00822.x.

Pećina-Šlaus, N. 2003. Tumor suppressor gene E-cadherin and its role in normal and malignant cells. *Cancer Cell Int.* 3:17. doi:10.1186/1475-2867-3-17.

Peddi, P.F., M.J. Ellis, and C. Ma. 2011. Molecular Basis of Triple Negative Breast Cancer and Implications for Therapy. *Int. J. Breast Cancer*. 2012. doi:10.1155/2012/217185.

Pellet-Many, C., P. Frankel, H. Jia, and I. Zachary. 2008. Neuropilins: structure, function and role in disease. *Biochem. J.* 411:211–226. doi:10.1042/BJ20071639.

Perou, C.M., T. Sørlie, M.B. Eisen, M. van de Rijn, S.S. Jeffrey, C.A. Rees, J.R. Pollack, D.T. Ross, H. Johnsen, L.A. Akslen, O. Fluge, A. Pergamenschikov, C. Williams, S.X. Zhu, P.E. Lønning, A.L. Børresen-Dale, P.O. Brown, and D. Botstein. 2000. Molecular portraits of human breast tumours. *Nature*. 406:747–752. doi:10.1038/35021093.

Petit, T. 2004. [Anthracycline-induced cardiotoxicity]. *Bull. Cancer (Paris)*. 91 Suppl 3:159–165.

Petti, L.M., P.M. Irusta, and D. DiMaio. 1998. Oncogenic activation of the PDGF beta receptor by the transmembrane domain of p185neu*. *Oncogene*. 16:843–851. doi:10.1038/sj.onc.1201590.

Popot, J.-L., T. Althoff, D. Bagnard, J.-L. Banères, P. Bazzacco, E. Billon-Denis, L.J. Catoire, P. Champeil, D. Charvolin, M.J. Cocco, G. Crémel, T. Dahmane, L.M. de la Maza, C. Ebel, F. Gabel, F. Giusti, Y. Gohon, E. Goormaghtigh, E. Guittet, J.H. Kleinschmidt, W. Kühlbrandt, C. Le Bon, K.L. Martinez, M. Picard, B. Pucci, J.N. Sachs, C. Tribet, C. van Heijenoort, F. Wien, F. Zito, and M. Zoonens. 2011. Amphipols from A to Z. *Annu. Rev. Biophys.* 40:379–408. doi:10.1146/annurev-biophys-042910-155219.

Popot, J.-L., E.A. Berry, D. Charvolin, C. Creuzenet, C. Ebel, D.M. Engelman, M. Flötenmeyer, F. Giusti, Y. Gohon, Q. Hong, J.H. Lakey, K. Leonard, H.A. Shuman, P. Timmins, D.E. Warschawski, F. Zito, M. Zoonens, B. Pucci,

and C. Tribet. 2003. Amphipols: polymeric surfactants for membrane biology research. *Cell. Mol. Life Sci. CMLS*. 60:1559–1574.

Prahst, C., M. Héroult, A.A. Lanahan, N. Uziel, O. Kessler, N. Shraga-Heled, M. Simons, G. Neufeld, and H.G. Augustin. 2008. Neuropilin-1-VEGFR-2 complexing requires the PDZ-binding domain of neuropilin-1. *J. Biol. Chem.* 283:25110–25114. doi:10.1074/jbc.C800137200.

Prat, A., J.S. Parker, O. Karginova, C. Fan, C. Livasy, J.I. Herschkowitz, X. He, and C.M. Perou. 2010. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res.* 12:R68. doi:10.1186/bcr2635.

Prat, A., and C.M. Perou. 2011. Deconstructing the molecular portraits of breast cancer. *Mol. Oncol.* 5:5–23. doi:10.1016/j.molonc.2010.11.003.

Raica, M., I. Jung, A.M. Cîmpean, C. Suciu, and A.M. Mureşan. 2009. From conventional pathologic diagnosis to the molecular classification of breast carcinoma: are we ready for the change? *Romanian J. Morphol. Embryol. Rev. Roum. Morphol. Embryol.* 50:5–13.

Reznikoff, W.S. 1992. The lactose operon-controlling elements: a complex paradigm. *Mol. Microbiol.* 6:2419–2422.

Roberts, P.J., J.E. Usary, D.B. Darr, P.M. Dillon, A.D. Pfefferle, M.C. Whittle, J.S. Duncan, S.M. Johnson, A.J. Combest, J. Jin, W.C. Zamboni, G.L. Johnson, C.M. Perou, and N.E. Sharpless. 2012. Combined PI3K/mTOR and MEK inhibition provides broad antitumor activity in faithful murine cancer models. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* 18:5290–5303. doi:10.1158/1078-0432.CCR-12-0563.

Robson, D., and S. Verma. 2009. Anthracyclines in Early-Stage Breast Cancer: Is It the End of an Era? *The Oncologist*. 14:950–958. doi:10.1634/theoncologist.2008-0070.

Rossignol, M., M.L. Gagnon, and M. Klagsbrun. 2000a. Genomic organization of human neuropilin-1 and neuropilin-2 genes: identification and distribution of splice variants and soluble isoforms. *Genomics*. 70:211–222. doi:10.1006/geno.2000.6381.

Rossignol, M., M.L. Gagnon, and M. Klagsbrun. 2000b. Genomic Organization of Human Neuropilin-1 and Neuropilin-2 Genes: Identification and Distribution of Splice Variants and Soluble Isoforms. *Genomics*. 70:211–222. doi:10.1006/geno.2000.6381.

Roth, L., C. Nasarre, S. Dirrig-Grosch, D. Aunis, G. Crémel, P. Hubert, and D. Bagnard. 2008. Transmembrane domain interactions control biological functions of neuropilin-1. *Mol. Biol. Cell*. 19:646–654. doi:10.1091/mbc.E07-06-0625.

Rowse, G.J., S.R. Ritland, and S.J. Gendler. 1998. Genetic modulation of neu proto-oncogene-induced mammary tumorigenesis. *Cancer Res.* 58:2675–2679.

Ruan, W., E. Lindner, and D. Langosch. 2004. The interface of a membrane-spanning leucine zipper mapped by asparagine-scanning mutagenesis. *Protein Sci. Publ. Protein Soc.* 13:555–559. doi:10.1110/ps.03357404.

Russ, W.P., and D.M. Engelman. 1999. TOXCAT: a measure of transmembrane helix association in a biological membrane. *Proc. Natl. Acad. Sci. U. S. A.* 96:863–868.

Russell, K.S., and M.C. Hung. 1992. Transcriptional repression of the neu protooncogene by estrogen stimulated estrogen receptor. *Cancer Res.* 52:6624–6629.

Sahai, E. 2007. Illuminating the metastatic process. *Nat. Rev. Cancer*. 7:737–749. doi:10.1038/nrc2229.

Sal-Man, N., D. Gerber, I. Bloch, and Y. Shai. 2007. Specificity in Transmembrane Helix-Helix Interactions Mediated by Aromatic Residues. *J. Biol. Chem.* 282:19753–19761. doi:10.1074/jbc.M610368200.

Scarlato, M., J. Ara, P. Bannerman, S. Scherer, and D. Pleasure. 2003. Induction of neuropilins-1 and -2 and their ligands, Sema3A, Sema3F, and VEGF, during Wallerian degeneration in the peripheral nervous system. *Exp. Neurol.* 183:489–498.

Schairer, C., P.J. Mink, L. Carroll, and S.S. Devesa. 2004. Probabilities of death from breast cancer and other causes among female breast cancer patients. *J. Natl. Cancer Inst.* 96:1311–1321. doi:10.1093/jnci/djh253.

Schechter, A.L., D.F. Stern, L. Vaidyanathan, S.J. Decker, J.A. Drebin, M.I. Greene, and R.A. Weinberg. 1984. The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. *Nature*. 312:513–516. doi:10.1038/312513a0.

Schneider, D., and D.M. Engelman. 2003. GALLEX, a Measurement of Heterologous Association of Transmembrane Helices in a Biological Membrane. *J. Biol. Chem.* 278:3105–3111. doi:10.1074/jbc.M206287200.

Senes, A., M. Gerstein, and D.M. Engelman. 2000. Statistical analysis of amino acid patterns in transmembrane helices: the GxxxG motif occurs frequently and in association with beta-branched residues at neighboring positions. *J. Mol. Biol.* 296:921–936. doi:10.1006/jmbi.1999.3488.

Shalaby, F., J. Rossant, T.P. Yamaguchi, M. Gertsenstein, X.F. Wu, M.L. Breitman, and A.C. Schuh. 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature*. 376:62–66. doi:10.1038/376062a0.

Shiang, R., L.M. Thompson, Y.Z. Zhu, D.M. Church, T.J. Fielder, M. Bocian, S.T. Winokur, and J.J. Wasmuth. 1994. Mutations in the transmembrane domain of FGFR3 cause the most common genetic form of dwarfism, achondroplasia. *Cell*. 78:335–342.

Shih, C., L.C. Padhy, M. Murray, and R.A. Weinberg. 1981. Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature*. 290:261–264.

Siegel, P.M., E.D. Ryan, R.D. Cardiff, and W.J. Muller. 1999. Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. *EMBO J.* 18:2149–2164. doi:10.1093/emboj/18.8.2149.

Solomayer, E.F., I.J. Diel, G.C. Meyberg, C. Gollan, and G. Bastert. 2000. Metastatic breast cancer: clinical course, prognosis and therapy related to the first site of metastasis. *Breast Cancer Res. Treat.* 59:271–278.

Starzec, A., R. Vassy, A. Martin, M. Lecouvey, M. Di Benedetto, M. Crépin, and G.Y. Perret. 2006. Antiangiogenic and antitumor activities of peptide inhibiting the vascular endothelial growth factor binding to neuropilin-1. *Life Sci.* 79:2370–2381. doi:10.1016/j.lfs.2006.08.005.

Stegemann, S., F. Leveiller, D. Franchi, H. de Jong, and H. Lindén. 2007. When poor solubility becomes an issue: from early stage to proof of concept. *Eur. J. Pharm. Sci. Off. J. Eur. Fed. Pharm. Sci.* 31:249–261. doi:10.1016/j.ejps.2007.05.110.

Stephenson, J.M., S. Banerjee, N.K. Saxena, R. Cherian, and S.K. Banerjee. 2002. Neuropilin-1 is differentially expressed in myoepithelial cells and vascular smooth muscle cells in preneoplastic and neoplastic human breast: a possible marker for the progression of breast cancer. *Int. J. Cancer J. Int. Cancer*. 101:409–414. doi:10.1002/ijc.10611.

Stern, D.F., M.P. Kamps, and H. Cao. 1988. Oncogenic activation of p185neu stimulates tyrosine phosphorylation in vivo. *Mol. Cell. Biol.* 8:3969–3973.

Suto, F., K. Ito, M. Uemura, M. Shimizu, Y. Shinkawa, M. Sanbo, T. Shinoda, M. Tsuboi, S. Takashima, T. Yagi, and H. Fujisawa. 2005. Plexin-a4 mediates axon-repulsive activities of both secreted and transmembrane semaphorins and plays roles in nerve fiber guidance. *J. Neurosci. Off. J. Soc. Neurosci.* 25:3628–3637. doi:10.1523/JNEUROSCI.4480-04.2005.

Swain, S.M. 2011. Chemotherapy: updates and new perspectives. *Oncologist*. 16 Suppl 1:30–39. doi:10.1634/theoncologist.2011-S1-30.

Tai, W., R. Mahato, and K. Cheng. 2010. The role of HER2 in cancer therapy and targeted drug delivery. *J. Control. Release Off. J. Control. Release Soc.* 146:264–275. doi:10.1016/j.jconrel.2010.04.009.

Takagi, S., Y. Kasuya, M. Shimizu, T. Matsuura, M. Tsuboi, A. Kawakami, and H. Fujisawa. 1995. Expression of a cell adhesion molecule, neuropilin, in the developing chick nervous system. *Dev. Biol.* 170:207–222. doi:10.1006/dbio.1995.1208.

Takashima, S., M. Kitakaze, M. Asakura, H. Asanuma, S. Sanada, F. Tashiro, H. Niwa, J. Miyazaki Ji, S. Hirota, Y. Kitamura, T. Kitsukawa, H. Fujisawa, M. Klagsbrun, and M. Hori. 2002. Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 99:3657–3662. doi:10.1073/pnas.022017899.

Taneja, P., D.P. Frazier, R.D. Kendig, D. Maglic, T. Sugiyama, F. Kai, N.K. Taneja, and K. Inoue. 2009a. MMTV mouse models and the diagnostic values of MMTV-like sequences in human breast cancer. *Expert Rev. Mol. Diagn.* 9:423–440. doi:10.1586/ERM.09.31.

Taneja, P., D.P. Frazier, R.D. Kendig, D. Maglic, T. Sugiyama, F. Kai, N.K. Taneja, and K. Inoue. 2009b. MMTV mouse models and the diagnostic values of MMTV-like sequences in human breast cancer. *Expert Rev. Mol. Diagn.* 9:423–440. doi:10.1586/ERM.09.31.

Tanner, K.G., and J. Kyte. 1999. Dimerization of the extracellular domain of the receptor for epidermal growth factor containing the membrane-spanning segment in response to treatment with epidermal growth factor. *J. Biol. Chem.* 274:35985–35990.

Therasse, P., S.G. Arbuck, E.A. Eisenhauer, J. Wanders, R.S. Kaplan, L. Rubinstein, J. Verweij, M. Van Glabbeke, A.T. van Oosterom, M.C. Christian, and S.G. Gwyther. 2000. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J. Natl. Cancer Inst.* 92:205–216.

Turner, N.C., and A.L. Jones. 2008. Management of breast cancer--Part II. *BMJ*. 337:a540.

Verlinden, L., C. Kriebitzsch, I. Beullens, B.K. Tan, G. Carmeliet, and A. Verstuyf. 2013. Nrp2 deficiency leads to trabecular bone loss and is accompanied by enhanced osteoclast and reduced osteoblast numbers. *Bone*. 55:465–475. doi:10.1016/j.bone.2013.03.023.

Visvader, J.E. 2009. Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. *Genes Dev.* 23:2563–2577. doi:10.1101/gad.1849509.

Wan, J., P. Sazani, and R. Kole. 2009. Modification of HER2 pre-mRNA alternative splicing and its effects on breast cancer cells. *Int. J. Cancer J. Int. Cancer*. 124:772–777. doi:10.1002/ijc.24052.

Wang, S.-C., H.-C. Lien, W. Xia, I.-F. Chen, H.-W. Lo, Z. Wang, M. Ali-Seyed, D.-F. Lee, G. Bartholomeusz, F. Ou-Yang, D.K. Giri, and M.-C. Hung. 2004. Binding at and transactivation of the COX-2 promoter by nuclear tyrosine kinase receptor ErbB-2. *Cancer Cell*. 6:251–261. doi:10.1016/j.ccr.2004.07.012.

Wang, T.C., R.D. Cardiff, L. Zukerberg, E. Lees, A. Arnold, and E.V. Schmidt. 1994. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. *Nature*. 369:669–671. doi:10.1038/369669a0.

Watson, C.J., and W.T. Khaled. 2008. Mammary development in the embryo and adult: a journey of morphogenesis and commitment. *Development*. 135:995–1003. doi:10.1242/dev.005439.

Wei, P., X. Liu, M.-H. Hu, L.-M. Zuo, M. Kai, R. Wang, and S.-Z. Luo. 2011. The dimerization interface of the glycoprotein Ib? transmembrane domain corresponds to polar residues within a leucine zipper motif. *Protein Sci. Publ. Protein Soc.* 20:1814–1823. doi:10.1002/pro.713.

Weiner, D.B., Y. Kokai, T. Wada, J.A. Cohen, W.V. Williams, and M.I. Greene. 1989a. Linkage of tyrosine kinase activity with transforming ability of the p185neu oncoprotein. *Oncogene*. 4:1175–1183.

Weiner, D.B., J. Liu, J.A. Cohen, W.V. Williams, and M.I. Greene. 1989b. A point mutation in the neu oncogene mimics ligand induction of receptor aggregation. *Nature*. 339:230–231. doi:10.1038/339230a0.

Wellings, S.R., and H.M. Jensen. 1973. On the origin and progression of ductal carcinoma in the human breast. *J. Natl. Cancer Inst.* 50:1111–1118.

Wicki, A., and C. Rochlitz. 2012. Targeted therapies in breast cancer. *Swiss Med. Wkly.* 142:w13550. doi:10.4414/smw.2012.13550.

Wouters, F.S., P.J. Verveer, and P.I. Bastiaens. 2001. Imaging biochemistry inside cells. *Trends Cell Biol.* 11:203–211.

Xie, D., X.O. Shu, Z. Deng, W.Q. Wen, K.E. Creek, Q. Dai, Y.T. Gao, F. Jin, and W. Zheng. 2000. Population-based, case-control study of HER2 genetic polymorphism and breast cancer risk. *J. Natl. Cancer Inst.* 92:412–417.

Yamagishi, H., E.N. Olson, and D. Srivastava. 2000. The basic helix-loop-helix transcription factor, dHAND, is required for vascular development. *J. Clin. Invest.* 105:261–270. doi:10.1172/JCI8856.

Yang, H., M. Li, H. Chai, S. Yan, R. Zhang, Q. Yao, and C. Chen. 2004. Expression and regulation of neuropilins and VEGF receptors by TNF-alpha in human endothelial cells. *J. Surg. Res.* 122:249–255. doi:10.1016/j.jss.2004.05.007.

Yarden, Y. 2001. Biology of HER2 and its importance in breast cancer. *Oncology*. 61 Suppl 2:1–13. doi:55396.

Yared, J.A., and K.H.R. Tkaczuk. 2012. Update on taxane development: new analogs and new formulations. *Drug Des. Devel. Ther.* 6:371–384. doi:10.2147/DDDT.S28997.

Yasuoka, H., R. Kodama, M. Tsujimoto, K. Yoshidome, H. Akamatsu, M. Nakahara, M. Inagaki, T. Sanke, and Y. Nakamura. 2009. Neuropilin-2 expression in breast cancer: correlation with lymph node metastasis, poor prognosis, and regulation of CXCR4 expression. *BMC Cancer*. 9:220. doi:10.1186/1471-2407-9-220.

Yin, H., R.I. Litvinov, G. Vilaire, H. Zhu, W. Li, G.A. Caputo, D.T. Moore, J.D. Lear, J.W. Weisel, W.F. Degrado, and J.S. Bennett. 2006. Activation of platelet alphaIIbbeta3 by an exogenous peptide corresponding to the transmembrane domain of alphaIIb. *J. Biol. Chem.* 281:36732–36741. doi:10.1074/jbc.M605877200.

You, L.-R., F.-J. Lin, C.T. Lee, F.J. DeMayo, M.-J. Tsai, and S.Y. Tsai. 2005. Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. *Nature*. 435:98–104. doi:10.1038/nature03511.

Yuan, L., D. Moyon, L. Pardanaud, C. Bréant, M.J. Karkkainen, K. Alitalo, and A. Eichmann. 2002. Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Dev. Camb. Engl.* 129:4797–4806.