Elucidating novel molecular and cellular mechanisms in Tenascin-C dependent breast cancer aggressiveness

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Identification et caractérisation de nouveaux mécanismes moléculaires et cellulaires dépendant de la Ténascine-C et impliqués dans l'agressivité du cancer du sein

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“The greatest enemy of knowledge is not ignorance,

is the illusion of knowledge” (SH)

“Nuestra fuerza es el espíritu…”

Rubén Bonifaz Nuño

SUMMARY IN FRENCH

Identification et caractérisation de nouveaux mécanismes moléculaires et cellulaires dépendant de la Ténascine-C et impliqués dans l'agressivité du cancer du sein

Résumé de thèse d'Inés VELÁZQUEZ QUESADA Inserm U1109 - Université de Strasbourg

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1. Introduction

Le microenvironnement tumoral joue des rôles cruciaux tout au long de la formation et de la progression tumorales[1,2]. Celui-ci comprend la matrice extracellulaire (MEC)[3], dont la Ténascine-C (TNC) est un composant majeur très fortement exprimé dans différents types tumoraux, notamment les cancers du sein[4]. Pour les patientes atteintes de cancer du sein, les niveaux d'expression de la TNC corrèlent avec une évolution péjorative, la résistance au tamoxifène et la formation de métastases pulmonaires[5-8].

Au niveau mécanistique, la TNC exerce des effets pléiotropiques, en favorisant la survie, la prolifération et l'invasion des cellules tumorales, mais également l'angiogenèse, l'inflammation et la formation de métastases pulmonaires[5-8]. Le rôle potentiel de la TNC dans la progression tumorale mammaire a été précédemment analysé en utilisant le modèle transgénique MMTV-PymT[9] exprimant ou non la TNC[10]. L'absence de TNC ne modifie pas la progression tumorale, bien qu'une organisation tumorale altérée et un infiltrat de macrophages aient été notés[10]. En utilisant des modèles murins immuno-déprimés xénogreffés avec des cellules tumorales humaines, il a pu être montré que la TNC promeut la formation de métastases pulmonaires : en effet, les cellules cancéreuses mammaires exprimant la TNC produisent plus de métastases que celles n'exprimant pas la TNC[8, 11]. Récemment, une étude a révélé que ceci est dû à l'activation des voies de signalisation Wnt et Notch, augmentant ainsi la survie des cellules tumorales durant les phases précoces de la colonisation métastatique du poumon[8]. Par ailleurs, une autre étude a montré que la TNC produite par les cellules stromales positives pour le marqueur S100A4 (S100A+) est cruciale pour la croissance des métastases pulmonaires[12].

Le but principal de ma thèse était de mieux comprendre les différentes contributions de la TNC durant l'établissement des cancers mammaires et leur progression jusqu'au stade métastatique. Dans cette optique, mon projet a été basé sur l'utilisation de deux modèles transgéniques murins de la carcinogenèse mammaire induite par l'expression tissu-spécifique d'oncogènes : le modèle "PymT" (MMTV-PyMT, sur-exprimant l'antigène « T moyen » du virus du polyome ou « *Polyoma virus-middle T* »)[9] et le modèle "NeuNT" (MMTV-NeuNT ; surexprimant une version constitutivement active de *Neu*, l'homologue chez le rat de l'oncogène *ERBB2*/*HER2*)[13].

Dans les deux cas, les souris femelles développent des adénocarcinomes mammaires canalaires (de type luminal) mulitfocaux qui métastasent au niveau du poumon.

En utilisant ces deux modèles, les objectifs spécifiques de mon doctorat étaient les suivants :

Objectif 1 : Analyser les effets de la surexpression de la TNC sur la progression tumorale mammaire.

Objectif 2 : Analyser les effets de l'absence de la TNC sur la progression tumorale mammaire.

Objectif 3 : Identifier et valider de nouveaux médiateurs de la progression tumorale mammaire dépendants de la TNC et ayant une potentielle pertinence clinique.

2. Résultats

A. Objectif 1 : Analyser les effets de la surexpression de la TNC sur la progression tumorale mammaire

Des souris transgéniques exprimant la TNC sous le contrôle du promoteur MMTV avaient été générées précédemment au laboratoire dans le fond génétique FVB. Quatre fondateurs indépendants, surexprimant à des niveaux différents le transgène, ont été obtenus et validé. Les effets de l'expression ectopique de la TNC sur la progression tumorale ont été analysés après croisement de ces souris avec les modèles transgéniques des cancers mammaires (PyMT ou NeuNT). Pour chaque modèle, j'ai analysé des cohortes d'animaux dérivés de plusieurs fondateurs différents. En bref, mes nombreuses analyses ont révélé que la surexpression de la TNC ne modifiait aucun des paramètres analysés au niveau des tumeurs primaires (temps de latence d'apparition, nombre et masse totale des tumeurs) et des analyses histologiques n'ont pas non plus révélé de différences. De plus, la surexpression de la TNC n'a pas eu d'impact sur la formation de métastases pulmonaires. En résumé, je n'ai donc pas pu observer d'effet significatif de la surexpression de la TNC sur la progression tumorale mammaire.

B. Objectif 2 : Analyser les effets de l'absence de la TNC sur la progression tumorale mammaire

Une approche complémentaire pour l'étude des contributions de la TNC à la progression tumorale mammaire consiste à utiliser des souris présentant une invalidation génétique de la TNC (souris « knockout » pour le gène codant pour la TNC, ou TNCKO) Alors que les souris NeuNT et PymT sont dans un fond génétique FVB, les souris TNCKO[14] ont été obtenues dans un fond génétique SL129. Comme une étude précédente utilisant le modèle PyMT dans un fond génétique mixte n'a pas révélé d'effet de l'ablation de la TNC, nous avons décidé de travailler dans un fond génétique pur (FVB). Ainsi, j'ai donc au préalable dû effectuer des rétro-croisements des animaux TNCKO avec des souris FVB pendant 10 générations, avant d'obtenir des animaux TNCKO en fond génétique FVB pur. Ces animaux ont ensuite été croisés avec les modèles murins de la progression tumorale mammaire précités afin d'obtenir les animaux expérimentaux TNC+/+ (animaux contrôles ayant deux allèles sauvages), TNC +/- (hétérozygotes ayant un seul allèle sauvage invalidé et un allèle invalidé) et TNC-/- (animaux complètement invalidés pour la TNC).

B.1. Modèle PyMT

Cette étude m'a permis de montrer que l'absence de la TNC n'a pas d'impact majeur ni sur la formation et la croissance des tumeurs primaires mammaires ni sur le développement des métastases. Mes résultats, obtenus dans un fond génétique pur, semblent ainsi confirmer les données acquises précédemment avec ce même modèle tumoral mais sur un fond génétique mixte[14].

B.2. Modèle NeuNT

B.2.a. Etude 1 : stade « final » de la tumorigenèse

Mes données acquises en utilisant le modèle NeuNT ont révélé que, tout comme les animaux contrôles, les souris invalidées pour la TNC (TNC-/-) développent des tumeurs primaires mammaires mais après une période de latence (temps requis pour que la première tumeur primaire soit palpable) rallongée comparée aux animaux contrôles (t_{50}=183 jours pour les souris contrôles TNC +/+, *versus* t_{50}=232 jours pour les souris TNC-/-). L'analyse de la masse tumorale totale au moment du sacrifice des animaux (soit 3 mois après la première palpation) n'a pas révélé de différence significative entre les différents génotypes, suggérant que l'absence de TNC n'a pas d'effet majeur sur la croissance tumorale une fois que les tumeurs sont constituées.

Durant mes premières analyses des métastases pulmonaires observées dans le modèle NeuNT, je me suis rendu compte qu'une proportion importante de ces métastases est localisée dans la vascularisation, sous la forme d'emboles tumorales. Cette observation est cohérente avec les descriptions précédentes des métastases pulmonaires dans différents modèles murins transgéniques du sein induits par différentes formes d'ERBB2[13-18]. Il est même possible que ces emboles constituent un mode de dissémination des cellules tumorales : elles peuvent en effet être observées dans la vascularisation péritumorale dans ces modèles murins[18], ce que nous avons également pu observer dans le modèle NeuNT. De plus, la présence de ces emboles tumorales dans les vaisseaux péritumoraux - un phénomène connu comme « invasion lymphovasculaire » ou « angioivasion » - est reportée de manière fréquente chez les patientes atteintes de cancer du sein (de 20 à 35% d'entre elles, et en particulier dans les cas ERBB2+) où elle représente un signe d'agressivité des tumeurs, étant corrélée à une évolution péjorative pour les patientes (survie, envahissement ganglionnaire et formation de métastases distantes)[19-22]. De manière importante, au niveau de la vascularisation pulmonaire, les cellules tumorales sont capables d'extravaser à partir de ces emboles vers le parenchyme pulmonaire suite à une EMT (Transition Epithelio-Mésenchymateuse) induite une signalisation dépendante du TGF-beta par exemple[17], démontrant que ces emboles peuvent constituer des stades métastatiques transitoires capables d'évolution. Bien que la mort cellulaire par apoptose soit relativement rarement observée dans ces emboles (analyses de l'expression de la caspase-3 clivée), j'ai pu montrer que ces emboles tumorales pulmonaires sont constituées non seulement de cellules tumorales qui prolifèrent (révélé par l'expression du marqueur Ki67), mais aussi, fréquemment, par plusieurs types de cellules stromales, notamment des cellules fibroblastiques/mésenchymateuses (S100A4+) et des cellules endothéliales (CD31+). De plus, j'ai pu observer des emboles de très grande taille, obstruant de manière quasiment complète la lumière de vaisseaux majeurs de la circulation pulmonaire. L'ensemble de ces observations et résultats supportent la notion que les emboles tumorales observées dans le modèle NeuNT sont des métastases capables de croître et d'évoluer. Finalement, j'ai également pu montrer que la TNC est exprimée à la fois dans les emboles et les métastases parenchymateuses. De manière intéressante, la TNC semble systématiquement exprimée et distribuée à la

périphérie des emboles, avec d'autres molécules de la MEC, telles que des laminines et la fibronectine, ce qui suggère qu'une capsule de MEC pourrait être impliquée dans l'arrêt des cellules tumorales/emboles circulants, et/ou avoir des propriétés protectrices pour les cellules tumorales disséminées dans la circulation. Dans l'ensemble, mes observations suggèrent fortement que la TNC pourrait exercer un rôle crucial durant la dissémination des cellules tumorales métastatiques, les phases précoces de la colonisation métastatique du poumon et la croissance des métastases.

L'analyse des métastases dans les animaux TNC+/+ et TNC-/- a révélé que le nombre total de métastases n'est pas significativement modifié dans les animaux TNC-/-. Toutefois, la proportion d'emboles (stades précoces/intravasculaires de métastases) est augmentée dans les animaux TNC-/-, suggérant que la TNC pourrait favoriser l'évolution des emboles pulmonaires vers la formation de métastases parenchymateuses. De plus j'ai utilisé une approche stéréologique pour quantifier au niveau histologique la taille (surface) des lésions métastatiques. Cette analyse m'a permis de montrer que l'ablation de la TNC réduit d'environ 10 fois en moyenne l'ampleur totale (surface cumulée) des lésions métastatiques. Ces données suggèrent que même si l'absence de TNC ne semble pas altérer la dissémination à partir des tumeurs primaires, la croissance et l'évolution des métastases pulmonaires sont fortement altérées. La caractérisation des mécanismes cellulaires mis en jeu est en cours (notamment par des analyses de la prolifération et de l'apoptose après marquages de tissus et quantifications dans ces métastases).

B.2.b. Etude 2 : stade précoce de la tumorigenèse

Un effet significatif de l'absence de TNC a été noté sur le temps de latence tumorale (déterminé par palpation) dans le modèle NeuNT. Afin de mieux caractériser la contribution de la TNC à ces tous premiers stades de l'initiation et de la progression tumorales, j'ai réalisé une seconde étude des conséquences de l'ablation de la TNC. Ainsi, les animaux ont été sacrifiés, avant que toute tumeur primaire ne soit détectable par palpation (soit à l'âge de 4.5 mois). A ce stade relativement précoce, après sacrifice des animaux, j'ai pu observer macroscopiquement dans quelques glandes mammaires des nodules tumoraux de petite taille (jusqu'à 5 à 6 mm de diamètre) et possédant un apport sanguin évident. Ces cohortes d'animaux ont été analysées de manière systématique, notamment en réalisant des « préparations entières » de la glande mammaire afin d'obtenir une vue complète du tissu et de pouvoir y quantifier les lésions tumorales précoces. Une variabilité importante de la ramification de l'arbre canalaire a été observée entre les animaux mais sans différence entre les groupes expérimentaux (TNC+/+, TNC+/- et TNC-/-). Après observation, j'ai quantifié le nombre et la taille (surface) de lésions précoces (dont la plus grande dimension dépassait les 200μm, seuil déterminé après examen de glandes mammaires d'animaux contrôles). De tels nodules ont été observés dans 63% des souris TNC+/+, 86% des souris TNC +/-, et 50% des animaux TNC-/-. Parmi ces lésions précoces, aucune différence n'a été notée pour les plus petites d'entre elles (surface inférieure à 0.2 mm^2). Toutefois, une diminution significative du nombre de lésions précoces de plus grande taille (surface supérieure à 0.2 mm^2) a été observée pour les animaux TNC-/-. Ces résultats suggèrent donc que l'absence de TNC entraîne un retard de la croissance tumorale initiale et ainsi semblent confirmer le retard d'apparition des tumeurs palpables observé dans les animaux TNC-/- lors de ma première étude. De manière plus intéressante, cette seconde étude permet une détermination des mécanismes mis en jeu,

notamment en réalisant des analyses histologiques. Notre étude préliminaire montre en effet que des lésions précoces (hyperplasies, carcinomes in situ) et des tumeurs de petite taille peuvent être observées sur des coupes de ces glandes mammaires. L'état hyperprolifératif des canaux/acini a été confirmé par des marquages Ki67, révélant que quasiment toutes les cellules épithéliales prolifèrent (contrairement à des glandes mammaires contrôles sans tumeur/expression d'oncogène). De plus, la TNC ne semble pas exprimée au niveau des nodules hyperplastiques (hyperprolifératifs) ni des carcinomes canalaires *in situ*, mais seulement au niveau des carcinomes infiltrants. Cette observation pourrait expliquer la diminution du nombre des lésions précoces de plus grande taille observée dans les animaux TNC-/-, suggérant ensemble que la TNC est importante pour promouvoir la progression des carcinomes *in situ* en carcinomes infiltrants de plus grande taille. A ce stade, la TNC forme des travées à l'intérieur du tissu tumoral, de manière similaire à ce qui peut être observé dans les tumeurs primaires mammaires de plus grande taille ou dans les métastases pulmonaires parenchymateuses.

En conclusion, mes résultats acquis avec le modèle transgénique NeuNT soutiennent fortement la notion que la TNC exerce des rôles pléiotropiques et cruciaux à la fois à des stades précoces (formation et croissance des tumeurs primaires) et à des stades ultimes (métastases pulmonaires) de la progression tumorale mammaire.

C. Etablissement de nouveaux modèles cellulaires syngéniques du cancer du sein (lié à l'objectif 3)

Les modèles murins génétiquement modifiés représentent des outils puissants pour l'étude du développement tumoral *in vivo*. Toutefois, leur mise en place et développement ainsi que le temps de latence jusqu'à l'apparition des tumeurs primaires peuvent prendre beaucoup de temps. Cela est particulièrement vrai pour le modèle NeuNT, pour lequel le temps de latence tumorale peut atteindre jusqu'à 200 jours pour les animaux contrôles et plus de 300 pour les animaux invalidés pour la TNC, et de plus 3 mois supplémentaires sont ensuite requis pour qu'une majorité des animaux développent des métastases pulmonaires. Afin de contourner ce problème, j'ai développé de nouvelles lignées cellulaires cancéreuses mammaires syngéniques à partir des deux modèles transgéniques utilisés au laboratoire (PyMT et NeuNT ; seul ce dernier est décrit ci-après, étant donné qu'il a été utilisé pour mes expériences *in vitro* et *in vivo*, en cohérence avec les phénotypes décrits ci-avant lors de mon étude des animaux transgéniques). Mon but était d'établir de nouvelles lignées cellulaires proliférant *in vitro* et qui sont tumorigéniques lorsque les cellules sont réimplantées *in vivo* dans des souris syngéniques (souris de même fond génétique, FVB, que le modèle transgénique d'origine) totalement immuno-compétentes. Dans ce but, après sacrifice d'un animal transgénique NeuNT (NeuNT193), une tumeur mammaire a été dissociée de manière mécanique et enzymatique afin de mettre en culture les cellules. Après plusieurs semaines et passage des cellules, une population pure de cellules épithéliales pu être isolée et une analyse immunocytochimique m'a permis de confirmer la nature épithéliale et tumorale de ces cellules, en révélant l'expression des marqueurs cytokératines 8/18 (marqueur épithélial luminal) et de l'oncoprotéine ERBB2. Afin de déterminer leur tumorigénicité, les cellules NeuNT193 ont été ensuite implantées dans des animaux syngéniques (FVB) de manière hétérotopique (injections sous-cutanées) ou orthotopique (injections dans la glande mammaire). De manière surprenante, les cellules injectées ont conduit à la formation de tumeurs primaires dans une très forte proportion des cas (>90% des cas).

Une analyse histologique conventionnelle m'a de plus permis de montrer que ces tumeurs primaires obtenues après injections des cellules sont phénotypiquement impossibles à distinguer des tumeurs observées dans les animaux transgéniques (observation confirmée par une pathologiste spécialiste des cancers mammaires). De manière très intéressante, j'ai de plus observé que les cellules tumorales implantées dans la glande mammaire produisent spontanément des métastases pulmonaires (dans 60% des souris), et que ces métastases récapitulent le patron observé dans le modèle transgénique (présence à la fois d'emboles tumorales dans la vasculature et de métastases parenchymateuses). De plus, le potentiel métastatique des cellules NeuNT193 a en parallèle été déterminé en injectant ces cellules directement dans la circulation sanguine, au niveau de la veine latérale de la queue des animaux, ce qui représente un test classiquement utilisé pour déterminer le potentiel de cellules à métastaser au niveau du poumon. La formation de métastases pulmonaires a également pu être observée, dans 50% des animaux injectés.

Un des buts premiers de la production de ces lignées était d'être capable de manipuler le niveau d'expression de la TNC et d'en analyser les conséquences. Ainsi, j'ai utilisé une approche basée sur des « shRNA » (TNC « knockdown » ou TNCKD) dont l'expression est médiée par transductions lentivirales afin de réduire l'expression de la TNC de manière stable. Une réduction satisfaisante du niveau d'expression de la TNC a pu être observée (en quantifiant l'expression de l'ARNm par RT-PCR quantitative et de la protéine par western blot) pour deux shRNAs indépendants. Des premiers tests fonctionnnels *in vitro* (d'autres tests sont encore en cours) m'ont permis de montrer que la réduction de l'expression de la TNC conduit à un retard de prolifération des cellules NeuNT193. Finalement, les cellules NeuNT193 contrôles et TNCKD ont été injectées de manière orthotopique *in vivo*, à des animaux contrôles (TNC+/+) ou à des animaux invalidés pour la TNC (-/-). De manière importante, cette étude (en cours) a une valeur ajoutée par rapport à l'étude précédente utilisant les modèles transgéniques car elle permettra de déterminer la contribution relative de la TNC produite par les cellules tumorales d'une part et par les cellules stromales d'autre part, sur l'initiation et la croissance de la tumeur primaire, mais aussi sur la formation de métastases pulmonaires.

Finalement, j'ai à nouveau isolé *in vitro* des cellules NeuNT193 à partir des métastases pulmonaires (lignées NeuNT193-LM, LM pour « Lung Metastasis ») survenues spontanément après implantation orthotopique (LM1 à 7) ou injection dans le veine de la queue des animaux (LM 8 à 11). Ces dérivés de la lignée NeuNT193 parentale devraient *a priori* être plus métastatiques que la lignée parentale (en cours de détermination), et ainsi représenter des outils de choix pour les études futures au laboratoire. Des dérivés des lignées NeuNT193 (parentale), LM1 et LM11 ont également été établis afin que les cellules expriment de manière stable un marqueur fluorescent et la luciferase. Dans le futur, ces nouveaux outils cellulaires vont permettre d'effectuer un suivi et la quantification de manière non-invasive *in vivo* de la croissance tumorale primaire, de la dissémination et la formation de métastases pulmonaires.

3. Conclusions

En utilisant des modèles murins et des approches *in vitro*, mon travail de doctorat a permis de mettre en lumière différentes contributions importantes de la TNC au cours de la carcinogenèse mammaire. Les perspectives directes de mes travaux sont nombreuses mais de manière générale les analyses encore en cours permettront rapidement de mieux cerner les mécanismes moléculaires et cellulaires mis en jeu en aval de la TNC et impliqués dans la progression tumorale mammaire. A plus long terme, ce travail pourrait permettre d'identifier de nouveaux marqueurs moléculaires de l'action de la TNC et d'évaluer leur pertinence clinique potentielle.

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ABSTRACT

Elucidating novel molecular and cellular mechanisms in Tenascin-C dependent breast cancer aggressiveness

Background

The microenvironment, which comprises the extracellular matrix (ECM), plays instrumental roles during tumor formation and progression. Tenascin-C (TNC) is a major ECM component highly expressed in breast cancer, correlating with poor prognosis, tamoxifen resistance and lung metastasis formation. TNC exerts pleiotropic effects by promoting tumor cell survival, proliferation and invasion as well as angiogenesis and inflammation. Finally, TNC expression correlates with lung metastagenicity and was shown to promote lung metastasis formation.

Objectives

The main goal of my thesis was to try to comprehensively understand the several contributions of TNC during breast cancer establishment and progression to metastatic disease. Using the MMTV-PyMT and the MMTV-NeuNT oncogene-induced models of breast cancer, my first objective was to analyze the effects of TNC overexpression and the absence of TNC on breast cancer progression *in vivo*.

The second objective of my thesis was to establish syngeneic cell lines from MMTV-PyMT- and MMTV-NeuNT-derived breast tumors in order to be able to analyze the role of TNC *in vitro* and *in vivo* in immune-competent FVB mice.

Results

We established the MMTV-mTNC model, to evaluate the effect of the overexpression of TNC in breast cancer progression but unfortunately did not find overexpression of TNC in NeuNT breast tumors. Therefore in this work I could not evaluate the effect of the overexpression of TNC in breast tumor formation. My analyses also revealed that the absence of TNC does not affect breast tumorigenesis in the MMTV-PyMT breast cancer mouse model, confirming a previous study .

More interestingly, using the MMTV-NeuNT model, we show that TNC promotes primary tumor initiation and lung metastatic colonization. In the lung, TNC increases of cancer cell survival in intravascular metastases and promotes their progression.

Finally, we established breast cancer cell lines from MMTV-PyMT and MMTV-NeuNT mouse models that grow *in vitro* and are tumorigenic when re-implanted in syngeneic, fully immuno-competent mice.

Conclusions

In this work we had shown that TNC participates in tumor initiation and in lung metastasis colonization in an ErbB2-driven transgenic breast cancer mouse model.

The established cell lines are alternative tools useful for *in vivo* and *in vitro* studies in breast cancer research.

This study participates to a better understanding of TNC contributions in breast cancer progression and may pave the way towards the identification of novel molecular and cellular mechanisms implicated.

SCIENTIFIC CONTRIBUTIONS

(Written by Gertraud Orend, PhD supervisor)

The results of this thesis have not yet been published but three manuscripts are in preparation for submission of publication:

- **Tenascin-C promotes early and late events in ErbB2 driven lung metastasis**

Ines Velazquez-Quesada, Martial Kammerer, Christiane Arnold, Michael van der Heyden, Thomas Hussenet* and Gertraud Orend*, * co-corresponding authors

- **Generation of a novel cellular tool for drug discovery research in breast cancer**

Ines Velazquez-Quesada, Christiane Arnold, Thomas Hussenet* and Gertraud Orend*, * co-corresponding authors

- **Transmembrane domain targeting peptide antagonizing ErbB2/neu inhibits breast tumor growth and metastasis**

Alexia Arpel, Paul Sawma, Caroline Spenlé, Norbert Garnier, Ines Velazquez, Thomas Hussenet, Samia Aci-Sèche, Jean-Pierre Duneau, Nadège Baumlin, Monique Genest, David Brasse, Pierre Hubert, Gérard Crémel, Patrice Laquerrière, Gertraud Orend and Dominique Bagnard*, * corresponding author.

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ABBREVIATIONS

ADH	Atypical ductal hyperplasia
ATP	Adenosine-5'-triphosphate
aa	Amino acids
cDNA	complementary DNA
CK	Cytokeratin
CSC	Cancer stem cells
CTC	Circulating tumor cells
DAB	3,3'-Diaminobenzidine
DCIS	Ductal carcinoma *in situ*
DNA	Deoxyribonucleic acid
DTC	Disseminated tumor cells
EC	Endothelial cells
ECM	Extracellular matrix
EGFL	Epidermal growth factor like
EGFR	Epithelial growth factor receptor
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
ErbB2 (Neu,HER2)	Epidermal growth factor receptor 2
FTIII	Fibronectin type-III
GEM	Genetically engineered mice
HE	Hematoxilin eosin
HER	Human epidermal growth factor receptor
HRP	Horseradish peroxidase
IBC	Invasive breast carcinoma
IDC	Invasive ductal carcinoma
IF	Immunofluoresence
IHC	Immunohistochemistry
KD	Knock-down
KO	Knock-out
LCIS	Lobular carcinoma *in situ*

MMTV	Mouse mammary tumor virus
MMTV-LTR (MMTV)	Mouse mammary tumor virus-long term repeat
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF-BB	Platelet-derived growth factor subunit B homodimer
PFA	Paraformaldehyde
POST	Periostin
PR	Progesterone receptor
PyMT (PyV-mT)	Polyomavirus middle T *gene*
RNA	Ribonucleic acid
RPL19	60S ribosomal protein L19
RT	Room temperature
RT-qPCR	Real-time quantitative polymerase chain reaction
SDS	Sodium dodecyl sulfate
SPARC	Secreted protein acidic and rich in cysteine
TNC	Tenascin C
VEGF	Vascular endothelial growth factor
waftd	Weeks after first tumor detection

INTRODUCTION

Cancer seems to be an old disease. Mummies from Egypt and Chile have been diagnosed with benign or malign tumors and bones dating from 14th-19th century showed also evidence of cancer (1). Furthermore, several scripts dating from the fifth century BCE to 1300 CE were written about cancer and it seems that even Greeks were able to distinguish between benign and malignant tumors (2).

The number of cancer diagnostics has increased in recent years. The World Health Organization suggests that this increase is due to 21st-century lifestyles including tobacco use, insufficient physical activity or unhealthy diets (3). Moreover, it has been proposed that the increase in the number of cancer cases is also due to a longer life expectancy and a better diagnosis of the diseases (2).

Cancer is a progressive disease, notably caused by the uncontrolled expansion of cells within a tissue and that at late stages are able to colonize distant tissues. Depending on the cell type of origin, a cancer can be classified as a carcinoma (epithelial origin), a sarcoma (stromal cell origin), a leukemia (blood cell origin), a lymphoma and a myeloma (immune cell origin) or a central nervous system cancer.

But how can normal cells become hyper-proliferative and form tumors? In normal conditions, tissues maintain an equilibrium in their number of cells by an intimate cell-cell and cell-stroma communication. These molecular and cellular crosstalks regulate different biological processes controlling cell fate within a tissue, such as proliferation or cell death. A tumor may start to form when tissue homeostasis is disrupted, leading to deregulated cellular interactions which, together with genetic modifications, can ultimately contribute to the transformation and progression of normal cells towards becoming highly proliferative-malignant cells. In 2000 and 2011 Douglas Hanahan and Robert Weinberg enumerated a list of skills that cancer cells commonly acquire during tumor formation and cancer progression (4)(5). These attributes include: proliferation-promoting signaling, evasion of growth-suppressor signals, cell death resistance, replicative immortality and metabolic reprogramming (4)(5). In addition and importantly, at several steps during tumor progression, tumor cells adapt to and shape their microenvironment, which is composed of both cells and the extracellular matrix (ECM) (4) (5) (6) (7). Among others, such crosstalks enable tumor cells to induce angiogenesis, to evade from the immune system and to become invasive and disseminate, a pre-requisite for metastasis formation (8).

1. HALLMARKS OF CANCER

1.1 Growth signal autonomy

In normal adult tissues, cell proliferation is regulated by external signals. This activation occurs when growth factors that are present in the microenvironment are sensed by cell membrane receptors and activate a cascade of intracellular events that finally trigger cell proliferation. Normally, this activation is highly regulated; but cancer cells develop different strategies to overcome such regulations. Thus in cancer cells the expression levels or the functional capabilities of different molecules involved in growth signaling pathways are modified, often by alterations at the level of cell membrane receptors. As examples, some cancer cells overexpress cell membrane receptors (e.g. ErbB2, EGFR) and thus become more responsive and sensitive to levels of their cognate ligand(s). Other tumor cells express a mutant, constitutively active, version of the receptor generating growth-factor independent activation of the downstream signaling pathway.

Downstream (intracellular) growth signaling is also frequently altered in cancer cells producing a constitutive activation of the pathway. Cancer cell proliferation can also be hyper-activated by a higher availability of growth factors secreted by cancer cells themselves or by tumor-associated stromal cells.

1.2 Evasion of anti-growth signals

Even after activation, cell proliferation is normally regulated at different levels. If one of these checkpoints fail, the cell cycle is blocked and the cell does not divide. Tumor suppressors are molecules whose function is to counteract abnormal cellular events that could lead to tumor formation. Some of them are regulators of cell proliferation and can block this cellular process if necessary. There are several kinds of tumor suppressor proteins; many of them participate in the regulation of the cell cycle and/or are involved in DNA repair. Selected well-known examples of such tumor suppressors, frequently inactivated in tumors, include pRb, p53, BRCA1 and BRCA2. Others participate in cell contact-inhibition mechanisms. In cancer cells, the expression, function and/or activation of proteins mediating anti-growth signals are often affected, hereby promoting cell proliferation.

1.3 Resisting cell death

Cells can die through diverse mechanisms including apoptosis and necrosis. Apoptosis is triggered in physiology, e.g. during embryogenesis, or pathological conditions. Apoptosis is activated by either external signals received through cell death membrane receptors or by internal signals produced by the release of pro-apoptotic molecules such as from mitochondria. These signals induce a sequence of events that include activation of caspases, DNA condensation and finally cell fragmentation that creates apoptotic bodies. Apoptosis is a process that does not cause inflammation because the cell membrane

remains intact, and the rest of the cell body is rapidly phagocytosed. Cancer cells escape death by apoptosis mainly by losing some of the activators, such as DNA damage sensors, or by increasing anti-apoptotic signals.

Whereas in apoptosis death is programmed, necrosis represents "cell death by accident". Cells that had suffered an extensive injury, e.g. by high physical/thermal injury, profound membrane damage, mitochondria dysfunction, or swelling of lysosomes enter an irreversible pathway that finally produces cell death (9). As a result of cell damage, lysosomal enzymes are released into the cytoplasm and degrade cellular components. The cell membrane is also damaged and cell permeability increases generating finally the destruction of the cell. In tumors cancer cells can die by necrosis releasing pro-inflammatory signals, which ironically promote tumor growth.

1.4 Unlimited replicative potential

Normal cells cannot replicate forever as they indeed can sense their own age by a "beautiful" molecular mechanism that depends on telomere shortening. In the early sixties, it was observed that cells have a finite lifespan. Almost 10 years later it was proposed that this is because after each cell division an end sequence of the chromosome called the telomere is not replicated during DNA replication and therefore becomes progressively shorter. When telomeres are too short, the cell cycle does not progress anymore (10). Cancer cells commonly evade this internal cellular control of aging mainly by two mechanisms: overexpression of telomerase or by alternative recombination-based telomere maintenance mechanisms.

1.5 Altered metabolism

One characteristic of cancer cells is that they use abnormal mechanisms to generate energy. Normal cells use glucose to obtain ATP. In aerobic conditions, the end product of glycolysis is pyruvate that enters the mitochondria to produce ATP. Otherwise, under low oxygen conditions cells trigger an anaerobic process where pyruvate is then metabolized into lactate. Cancer cells activate glycolysis even in the presence of oxygen, a process called aerobic glycolysis. Consequences of this process are the acidification of the tumor microenvironment because of the high concentration of secreted lactate and a high increase in the glucose consumption (11) (12).

1.6 Angiogenesis

The supply of oxygen and nutriments in tissues depends on a vascular system formed by blood vessels. In normal tissue blood vessels are composed of three layers: (a) the *tunica intima,* directly in contact with the lumen of the vessel and formed by a layer of endothelial cells (EC), a basement membrane and a thin layer of subendothelial connective tissue; (b) the *tunica media,* composed mainly by elastic fibers and

several layers of supporting smooth muscle cells; and (c) *the tunica adventitia,* made by loose connective tissue and other supporting cells like pericytes. The composition of these layers is different in arteries, veins and capillaries, and depends on the localization of the vessel and the internal pressure. Blood vessel assembly (vasculogenesis) occurs during embryonic development. In adult tissues, the formation of new blood vessels occurs from pre-existing ones by a process known as angiogenesis. Angiogenesis begins by the dilatation of a blood vessel in response to angiogenic signals. The basement membrane is locally degraded, endothelial tip cells sprout and invade the surrounding stroma. EC that are just behind the leading front (stalk cells) proliferate, form a migrating column and the lumen of the vessel and finally supporting cells are recruited (13).

Tumor growth and progression require persistent blood vessel formation. This happens because cancer cells produce and release pro-angiogenic factors that promote angiogenesis. Although vascularized, tumor blood vessels are structurally and functionally abnormal. In general one tumor can contain areas with different vessel density and vessels with different architecture e.g. wide or tortuous vessels, or vessels with an irregular lumen. Every layer of tumor vessels is abnormal: EC do not have a normal appearance, cells are not completely connected and sometimes they form multiple layers. In addition, arterial and venous identity is not well defined. The basement membrane composition and thickness is also abnormal, and there are few supporting cells covering tumor vessels. As a result, these vessels are leaky, providing poor oxygen and nutrient supply (14).

1.7 Evading of and subverting the immune system

Although the immune system is a barrier for tumor formation and progression, tumors can develop several mechanisms to exploit the immune system and to evade immune surveillance. One of these mechanisms includes the ability of tumor cells to thrive in a chronically inflamed microenvironment. Studies with xenograft and transgenic mouse models as well as epidemiological studies showed that inflammation produced by external or internal stimuli facilitates tumor formation and growth (15).

Tumor cells also have to evade immune recognition. The role of the immune system as surveillance of malignancy was proposed around 50 years ago by observing that immune-compromised mice are more prone to spontaneous or chemical induced tumor formation and that immune-deficient patients are more likely to develop tumors. The surveillance function of the immune system is mainly carried out by T and B cells, which recognize immunogenic antigens exposed in the cell membrane. Immune surveillance mechanisms limit tumor formation, but some tumor cells evade this by expressing few or none of these immunogenic proteins. It is thought that tumors developed in immune compromised mice are not exposed to this selection and contain highly immunogenic cells. Transplantation experiments of these tumor cells showed that they are inefficient at forming secondary tumors in a syngeneic host (4).

1.8 Invasion and metastasis

The main feature of cancer cells is their capability to disseminate and colonize the same or different organs in a multistep process called metastasis. The word metastasis is derived from a greek word meaning “displacement”. Dissemination begins when cancer cells cross the basement membrane, invade the surrounded stroma, and reach blood or lymph vessels. Then, metastatic cells are transported through blood or lymph vessels to the target organ(s) where they finally attach to the endothelium, extravasate and proliferate. As one can suppose metastasis requires a high plasticity of the cancer cells in order to go through the whole process since they have to adopt different features and survive in several new and hostile microenvironments. Even if the sequence of events that take cancer cells to metastasize is similar, particularities depending on the tumor cell, the primary tumor and the metastatic niche make metastasis a highly heterogeneous and overall inefficient process.

The invasion of the local stroma can occur in a single cell manner or collectively (7). During collective invasion, the invasive cells maintain expression of epithelial markers and cell-to-cell adhesions and only a subset of cells at the invasive front provides migratory grip pulling the rest of the cells. On the other hand, a single cell invasion implies dramatic molecular and cellular changes. The single cells that are able to detach from the primary tumor are those that lose some epithelial markers and acquire mesenchymal characteristics by a process known as epithelial mesenchymal transition (EMT), thus becoming more motile, more invasive and less proliferative. Single cells can also efficiently become invasive by acquiring amoeboid features. Cells with this morphology are round, do not have cell polarity, do not form stress fibers and do not contain focal adhesions (16). During amoeboid invasion cells weakly interact with the ECM and loose the capability to move towards a chemotactic gradient.

It is not well known how cancer cells intravasate, i.e. how they leave the tissue and enter into blood vessels. There are data suggesting that intravasation is an active process in which cancer cells migrate through blood vessels following nutrient or chemokine gradients. Other evidence proposes that cancer cells can be pushed to enter through the leaky tumor vessels in a passive manner(16) (17).

Although cancer cells can enter and travel in lymph vessels, and even the presence of cancer cells in regional lymph nodes is highly used as a prognostic marker, some data indicate that haematogenous circulation is the major route for metastatic dissemination (18). Single and aggregated circulating tumor cells (CTC) are often detectable in the blood of cancer patients (19)(20). Detection of these cells and analysis of their metastatic potential is a growing area in cancer research (19). Stromal cells as fibroblast can travel with the CTC, increasing their viability and their capability to form metastasis (21). Moreover, CTC can interact with platelets and circulating cells as leukocytes and this interaction enhances their survival (22) and invasiveness (23).

During their transit in the circulation, cancer cells interact with the vascular endothelium and some of them can dock onto it. Cancer cells can adhere to the endothelium by direct cell-cell interactions with

endothelial cells (24)(25). It might also be possible that the interaction between cancer cells and endothelium occurs in an indirect manner, e.g. via ECM molecules. There is indeed evidence that prior to extravasation, disseminated metastatic tumor cells can proliferate within the vasculature thus forming small colonies that later can destroy the vascular boundaries and outgrow in the parenchyma of the tissue (26) (27).

Many reports showed that cancer cells extravasate and invade the new tissue as solitary cells (28)(27) and hence the extravasation of cancer cells depends on the ability of the cell itself to invade (29)(30) and on the characteristics of the vessel walls. Interestingly, in 1998 Lapis and colleagues (1988) proposed an alternative invasion-independent mechanism of extravasation (31). Electron microscopic analysis of lungs recovered at different times after an intravenous injection of B16-F10 cells revealed two distinct of invasion-independent extravasation processes, whether extravasation occurred in lung capillaries or in lung arterioles. Immediately after lodging of the cancer cells to the capillaries a “fibrin reaction” occurred. Subsequently there is a retraction of the endothelium providing direct contact of tumor cells with the basement membrane. Endothelial cells appear to surround completely the cancer cells thus promoting their extravasation. The lodging of cancer cells to arterioles promotes also a rapid fibrin reaction. However, in arterioles, the endothelial layer does not retract. Instead, a new endothelial cell monolayer surrounds the tumor emboli. In these endothelialized emboli cancer cells proliferate until the lumen of the vessel is filled. Extravasation of this kind of metastasis occurs by mechanical disruption of the tumor clump. Even if molecular and cellular details of the extravasation process remain to be elucidated, it is obvious that it occurs by several mechanisms. Endothelialized tumor emboli have been observed also in primary tumors suggesting that this emboli can travel through the circulation while surrounded by a layer of endothelial cells (32)(33).

Extravasation of metastatic cells does not ensure colonization of the target organ. Even if cells succeed in colonization, this does not always occur immediately (34). Disseminated tumor cells (DTC) that will succeed in colonizing the target organ will first grow as micrometastatic colonies (potentially reaching an equilibrium between proliferating and apoptotic cells) and later form macrometastasis. Finally, DTC can also remain in a dormant state for years and even decades until the conditions of the new niche change and promote their proliferation (35) (36).

Since the beginning of studies dedicated to metastasis, it was evident that cancer cells do not metastasize to all organs, but they have favorite target tissues and that this preference depends of the origin of the primary tumor. For example, breast cancer cells metastasize mainly to lung, liver, brain and bone (37) (38), whereas the most important metastatic site of colon cancer is the liver. These affinities are not completely explained by the anatomical or mechanical characteristics of the circulation system, the metastasis formation requires also compatibility between cancer cells (the seed) and the metastatic

microenvironment (the soil) (39). Several studies have been done in order to identify genes that determine tissue-specific colonization, e.g. specific genes expressed by breast cancer cells that metastasize to lung and that are different from genes expressed by breast cancer cells that metastasize to brain. These subsets of genes are called metastatic signatures and vary from one metastatic target tissue to another (40)(41)(42). Although it is clear that diverse organs present different characteristics, it was thought that these characteristics do not change until the arrival of the cancer cells. The concept of the soil as a static participant has lately been challenged. David Lyden and colleagues demonstrated that cancer cells can prime the metastatic soil by releasing soluble factors to the circulation even before they escape from the primary tumor. Interestingly they observed that these factors are tissue-specific and determine the future targeted distant organ (43).

Overall, metastasis is a highly inefficient process. Although millions of cancer cells may shed in the circulation daily from a tumor (44), and cancer cells can disseminate from early steps of primary tumor formation (36), only a few of these cells are able to form metastasis due to several rate-limiting steps that include crucial tumor-stroma interactions in the early steps of metastatic colonization (18).

2. BREAST CARCINOMA

Breast cancer is by far the most common cancer diagnosed in women and the most common cause of death from cancer in women worldwide in 2008.

The female mammary gland is composed by a network of branched ducts that connect lobules (terminal ductules and acini) with the nipple **(Figure 1)**. Mature mammary ducts and lobules consist of an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells wrapped by a basement membrane. A supporting stroma, called the mammary fat pad, surrounds this tree of lobules and ducts. It contains extracellular matrix, blood vessels, nerves, adipocytes, fibroblast and immune cells. Breast carcinomas are produced by the malignancy of ductal or lobular epithelial cells of the breast **(Figure 1).** In women the risk to develop non-hereditary breast carcinoma is highly correlated with the age of the patient and different aspects of hormonal history like age at menarche, number of births and breast-feeding in addition to other factors generally related with cancer development like diet, obesity, exercise, environmental toxins and tobacco.

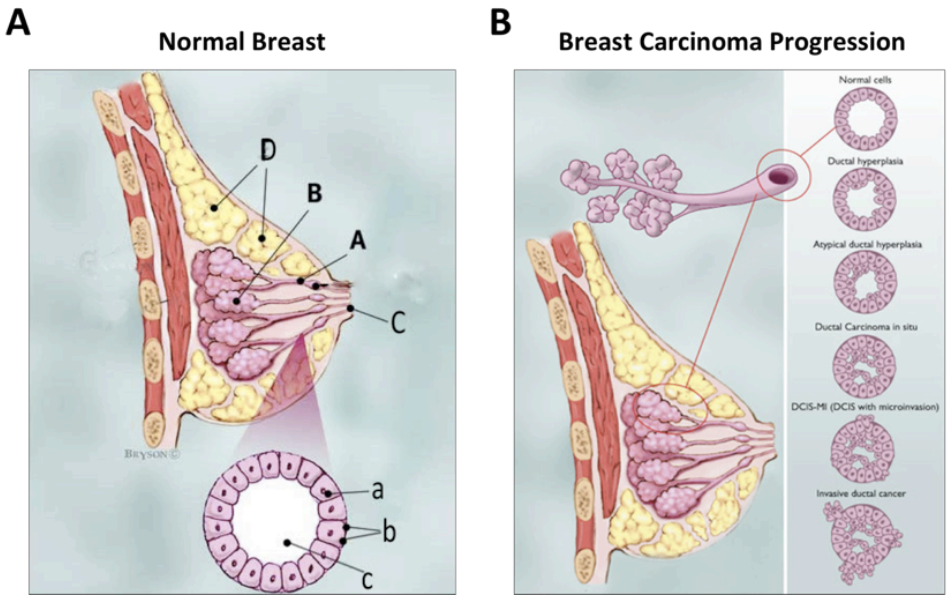


Figure 1. Normal breast tissue and progression to breast carcinoma.

(A) Normal breast is conformed by a network of ducts (A) that connect the nipple (C) with the lobules (B). This network is surrounded by a stroma constituted mainly by fat tissue (D). In enlargement its shown a lobule constituted by the lumen of the duct (c), epithelial cells (a) surrounded by a layer of myoepithelial cells and the base membrane (b).

(B) Breast carcinoma is a multistep process that starts with hyper-proliferation of epithelial cells and progress to invasive breast carcinoma.

Modified from the breast cancer organization internet site: http://www.breastcancer.org.

The diagnosis of breast cancer is higher in developed than in developing countries, albeit incidence of the latter has increased in the last decades, maybe due to changes in reproductive behavior, use of exogenous hormones and life style (45).

2.1 Breast cancer classification

Breast carcinoma is not a single disease, rather it comprises several subtypes with different biological characteristics and clinical outcome. Breast cancer is classified depending on several parameters, trying to group tumors with biological similarities, similar prognosis and treatment responses. I will briefly describe the different classification systems that exist and what they are based on.

2.1.1 Histological classification system

The histological classification of breast cancer groups tumors at a morphological level and depends on the microscopic anatomy of the tumor. Breast tumors can be classified as in situ carcinomas or invasive carcinomas. In situ carcinomas are either lobular (LCIS) or ductal (DCIS) which are further sub-classified according to the architectural appearance of the tumor as comedo, cribiform, micropapillary, papillary and solid. On the other hand, invasive carcinomas are further subdivided into six subgroups of which infiltrating ductal carcinoma (IDC) with 70-80% of all cases is the most common subtype (46).

2.1.2 Tumor grade

Histological grading determines the differentiation state of the tumor cells. This classification groups tumors as well (grade 1 or low grade), moderately (grade 2 or intermediate grade) and poorly differentiated (grade 3 or high grade) (47).

2.1.3 Tumor stage (TNM)

Gradation by tumor stage helps to determine the level of dissemination of a tumor and describes the size of the tumor (T), spreading of cancer cells to the lymph nodes (N) and the metastasis formation (M). Breast cancer is divided in five stages (from stage 0 to 4) in dependence of these parameters (47).

2.1.4 Receptor status

An important factor for prognosis and treatment of breast tumors is the expression of hormone receptors. The most important receptors involved in breast carcinogenesis are estrogen receptor (ER), progesterone receptor (PR) and ErbB2/HER2/Neu receptor. Determination of the receptor status is highly useful to choose the treatment(s), but is not enough to determine the breast tumor subtype or predict a prognosis (47).

2.1.5 Molecular breast cancer subtypes

The development of new technologies facilitated the analysis of a large number of genes in large collections of tumor samples. Global gene expression analysis provided a molecular classification of breast tumors. To date six breast adenocarcinoma subtypes have been identified (48) (49):

a. Luminal subtype A. Luminal tumors (subtype A and subtype B) are characterized by the expression of genes from the luminal epithelium as cytokeratin 8 (CK8) and 18 (CK18). Luminal subtype A tumors are considered as tumors in an early stage. Cancer cells look like normal epithelial breast cells, they express progesterone and estrogen receptors and are ErbB2 negative. Tumors are not highly proliferative and prognosis of patients is favorable.

b. Luminal subtype B. These tumors have a similar gene expression pattern than luminal subtype A tumors but they are highly proliferative. They have a more aggressive phenotype and correlate with a poor outcome.

c. Normal breast-like: This tumor-subtype expresses a large number of genes characteristic of adipose tissue and basal epithelial cells and expresses few genes characteristic of luminal cells. The clinical significance of these tumors is not clear due to the low number of cases analyzed.

d. Basal-like: These tumors present high expression levels of genes characteristic of myoepithelial cells like cytokeratin 5 (CK5), cytokeratin 6 (CK6) and cytokeratin 17 (CK17). One distinctive characteristic of this subtype is that they do not express ER, PR or ErbB2, because of which they are also called “triple negative”. Basal-like and triple negative are not synonymous and they should not be used interchangeably since normal breast-like and claudin-low carcinomas can also be triple negative tumors. Basal-like tumors are highly aggressive, they rapidly grow and present a poor prognosis.

e. Claudin-low: These tumors share some genetic characteristics with basal-like tumors as they are also triple-negative. A distinctive feature of these tumors is the low expression of genes that participate in tight junctions and cell-cell adhesion.

f. HER2^{+}: This tumor subtype is characterized by high expression levels of ErbB2 and genes associated with the Erbb2 pathway, as well as the low expression levels of ER. These tumors are highly proliferative. At the histological level, most of the ErbB2 overexpressing tumors develop intermediate to poorly differentiated ductal carcinomas (50). Usually HER2+ patients have a bad prognosis. However, the use of HER2 antibody therapy (Hercepin: trastuzumab) has improved patient survival. Moreover, recently a HER2-derived prognosis predictor (HDPP) was proposed. This signature sub-classifies HER2+ tumors in three subtypes with a different clinical outcome, but this signature is not yet widely used (51).

Each tumor subtype harbors different genetic alterations, has a different clinical outcome and responds differently to anti-cancer treatments (48). Although the analysis of gene expression profile of tumors is not practical as a routine technique, analysis of the expression of some "key" genes is useful to determine the molecular tumor subtypes (49) (52).

2.2 Breast cancer progression

The formation and progression of a breast carcinoma is a multi-step process with defined stages identified by histology. It is thought that It begins with a premalignant lesion called atypical ductal hyperplasia (ADH) that consists of hyper-proliferative epithelial ductal or lobular cells. Despite high levels of proliferation, ADH can considered as a benign lesion that does not always give rise to cancer (53). It is thought that ADH could progress into ductal carcinomas in situ (DCIS) or into lobular carcinomas in situ (LCIS) that are non-invasive lesions formed by abnormally proliferating cells. DCIS are already considered as a cancerous lesion, even if cells reside in their normal place and the basement membrane remains intact (54). If DCIS are not successfully treated, they can evolve into an invasive breast carcinoma (IBC). An IBC, also termed invasive duct carcinoma or invasive lobular carcinoma, is characterized by the presence of breast cancer cells in the surrounding stroma and an interrupted basement membrane. In this phase the risk to develop metastasis increases **(Figure 1)**. Breast carcinomas can spread through lymphatic vessels to regional and distant lymph nodes or disseminate through blood vessels. As mentioned breast cancer cells metastasize preferably to bone, brain, lung and liver (38) (37).

3. TRANSGENIC BREAST CANCER MOUSE MODELS

The first studies about cancer were based on retrospective analyses. Epidemiological correlations were made between the histology of the tumors and the prognosis of the patients. The development of cell biological methods favored the identification of genetic abnormalities associated with cancers. Amplification or overexpression of genes encoding regulators of proliferation and/or loss of heterozygosity of tumor suppressor genes (55) were observed. Breast cancer mouse models were developed to test the capability of these identified genes to induce tumor formation *in vivo*. Overall, two genetic strategies have been used to try recapitulating breast cancer in mice: overexpression of oncogenes (e.g. erbB2, myc and cyclinD1) and inactivation of tumor suppressors identified to be important in human breast tumors (e.g. trp53, brca1 and pten).

In the last years several breast cancer mouse models have been developed. Most of them overexpress breast cancer oncogenes (cox2, wnt1, erbB2/neu, tan, met, pymt) in mammary epithelial cells using cell type specific promoters (56). Some of these models were shown to mimic human breast tumor pathology at biological (57), histological (58) and molecular level (59) becoming a powerful tool to study breast cancer progression and to evaluate new potential cancer treatments.

3.1 Recapitulation of the human breast cancer disease

3.1.1 Recapitulation of histological characteristics of human breast tumors

Recently an extensive histological comparison of human and mouse breast tumors was done (57). Pathologists analyzed breast tumor samples from different genetically engineered mice (GEM) and human breast tumors. They observed: (a) some GEM tumors are similar to non-GEM mouse tumors, (b) many GEM produce tumors with unique transgene-specific characteristics, and (c) some GEM produce tumors that mimic human breast tumors. However, even if some GEM lesions closely mimic human breast tumors, they do not necessarily represent the entire tumor. It is interesting to note that higher similarities were observed in the poorly differentiated phenotypes.

3.1.2 Recapitulation of human breast cancer progression (from hyperproliferation to IBC)

It has been observed that most of the GEM mimic the multistep breast cancer process from hyperplasia to metastasis (60). Hyperplasia is defined as intraluminal proliferation of epithelial cells with or without cytological atypia and with an intact basement membrane. Atypical hyperplasias are the earlier lesions observed in mouse and human breast tissue (57)(61). These lesions evolve into DCIS and finally generate breast carcinomas. Human and mouse breast carcinomas produce similar morphological patterns of lesions and cellular events as cell proliferation, angiogenesis and immune cell recruitment. As in humans, mouse breast tumors can be invasive and metastatic. One important difference between murine and

human breast cancer is the organ preference of metastasis. Whereas breast cancer in humans metastasizes to bone, brain and lungs, most of the breast cancer mouse models metastasize to lung (37). Even if data showed that this difference could be determined by the cancer cells (34), the role of the metastatic microenvironment is not negligible.

3.1.3 Molecular players

Since expression of oncogenes identified in humans produce tumors in mice it is evident that human and mouse share some molecular key players. To determine the degree of molecular similarity Perou and colleagues compared genome wide gene expression profiles of murine breast tumors from several models with human breast tumors using microarrays (59). The first interesting conclusion of this analysis is that for some of the breast cancer mouse models obtained from different laboratories homogeneous expression patterns were observed, indicating consistency in the models. Moreover, mouse breast tumors showed expression profiles comparable to human breast tumors. Interestingly, even though different mouse models are comparable with distinct breast tumors, no single mouse model recapitulates all the expression features of a given human breast cancer subtype.

3.2 Mammary gland specific promoters

In order to express oncogenes in epithelial breast tissue in breast cancer mouse models the currently most used promoter is the MMTV-LTR promoter (56). The mouse mammary tumor virus-long term repeat (MMTV-LTR/MMTV) promoter is originally a DNA virus sequence that favors mammary tropism (62). This promoter is active in ductal and alveolar luminal epithelial and myoepithelial cells during all stages of mammary gland differentiation (63). Although the MMTV promoter is highly active in breast epithelial cells, it is also active in salivary glands, lungs, kidney and lymphoid tissues (64)(65). In fact, analysis of some breast cancer mouse models using the MMTV-LTR promoter, showed expression of the oncogene in the above mentioned non-mammary tissues (65)(66). Some of these mice developped hyperplasias in these tissues (65), but besides breast, any other tissue has developed tumors, thus suggesting that the expression of the oncogene or the levels of expression produced in other tissues are not sufficient to induce neoplastic transformation.

The MMTV-LTR contains in its sequence response elements to hormones, progesterone and dihydrostestosterone (67) the use of this promoter in breast cancer mouse models produced hormone-independent, ER-negative mammary tumors (67). It is possible that the hormone-independent characteristic is driven by epigenetic modifications (67).

3.3 MMTV-NeuNT model

One of the first oncogene used to produce breast tumors in mice was ErbB2. Studies in human breast cancer showed that ErbB2 is overexpressed in 25-30% of human breast tumors (68) mainly due to amplification of the gene (69). Currently, the expression of this gene is widely used as a prognostic marker and determines the choice of treatment (69).

ErbB2 also called HER2 (human) or Neu (rat) encodes a tyrosine kinase receptor which together with EGFR (HER1, erbB1), HER3 (erbB3) and HER4 (erbB4) comprise the human epidermal growth factor receptor family a.k.a ErbB or HER receptor family (68)(70)(71). In its general structure ErbB receptors contain an extracellular ligand-binding region, a transmembrane domain and a cytoplasmic tyrosine kinase domain **(Figure 2)** (72). Upon ligand binding, ErbB receptors undergo homo or hetero dimerization and transphosphorylation of their intracellular domains; this reaction is called activation of the receptor. Additionally, data reveals that dimers can suffer further oligomerisation conforming a complex signaling platform (71) (73).

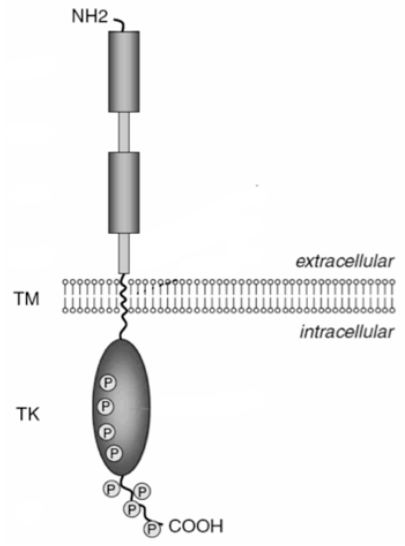


Figure 2. Structure of ErbB proteins

The structure or ErbB receptors consists of a N-terminal extracellular domain that contain ligand-binding regions, a short transmembrane domain (TM), and an intracellular catalytic tyrosine kinase domain (TK). Circled P indicates numerous sites of tyrosine phosphorylation within the intracellular domains.

Modified from Moasser MM; Oncogene 26; 2007.

Phosphorylation of specific sites of the receptors recruits cytoplasmic adaptor proteins or enzymes that trigger several intracellular pathways (68) (72). The ligand and the heterodimer partner determine the sites of phosphorylation and therefore the activated intracellular signaling (72). Since ErbB2 does not have a known ligand, it is mainly considered as a coreceptor (71) (72) (74). As such, ErbB2 can form heterodimers with several other receptors, including its major partners EGFR, ErbB3 (kinase-defective receptor) and ErbB4 (74). Several intracellular signaling pathways are triggered upon ErbB2 activation **(Figure 3)**. Ras and PLCg (phospholipase Cg) pathways are activated as a consequence of ErbB2-EGFR dimer formation. The PI3K/Akt pathway, required for ErbB2-driven cell transformation, can be activated

by the hetero dimerization of ErbB2 with EGFR or ErbB3. Whereas the Shc-activated mitogen–activated protein kinase (MAPK) pathway is activated by most active ErbB dimers (68) (72). This review shows that even though there is abundant information about the different proteins that participate in the ErbB signaling network, it is less clear what is the specific contribution of these molecules in transducing a proliferative, transforming and antiapoptotic signal (75).

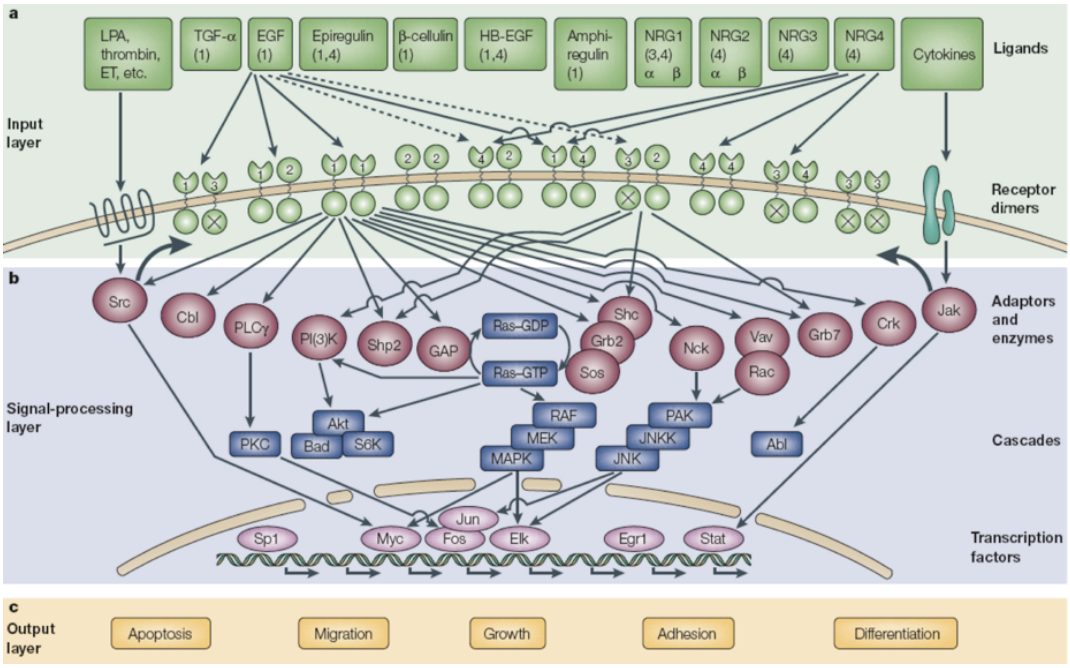


Figure 3. Simplified overview of the ErbB signaling network

(a) Ligands and the dimeric receptor combinations comprise the input layer. Numbers in each ligand lock indicate the respective high-affinity ErbB receptors. For simplicity, specificities of **receptor-bindings** are shown only for epidermal growth factor EGF) and neuregulin 4 (NRG4). ErbB2 binds no ligand with high affinity, and ErbB3 homodimers are catalytically inactive (crossed kinase domains).

(b) Signaling to the adaptor/enzyme layer is shown only for two receptor dimers: the weakly mitogenic ErbB1 homodimer, and the relatively potent ErbB2–ErbB3 heterodimer. Only some of the pathways and transcription factors are represented in this layer.

(c) Some of the possible outputs that promote tumor formation and cancer progression.

Taken from Yarden Y and Sliwkowski MX; Nature review 2; 2001.

Although several intracellular pathways can be activated *by* divers ErbB dimers, the potency and the kinetics of the signaling differ (72). It has been reported that the presence of ErbB2 in the dimer affects its kinetic. ErbB2 can decelerate ligand-receptor dissociation rates and increase the stability of the dimer, thus slowing internalization and reducing degradation (68) (74). As a result, ErbB2-comprising heterodimers trigger a prolonged and more potent signaling compared with the corresponding homo dimers **(Figure 4)** (68) (74) (75). It is possible that the overexpression of ErbB2 observed in breast cancer patients disrupts the equilibrium of the ErbB dimers, hence increasing the formation of ErbB2-comprising heterodimers and generating a signaling with higher biological potency (68) (74).

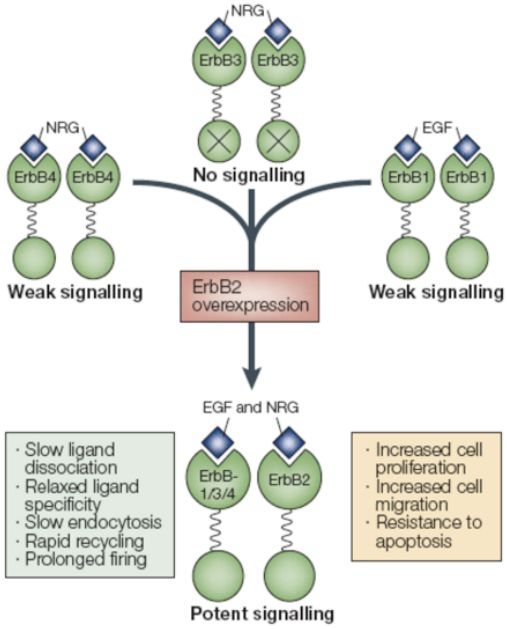


Figure 4. Signaling by ErbB homodimers in comparison with ErbB2-containing heterodimers

Receptors are shown as two lobes connected by a transmembrane stretch. Binding of a ligand (EGF-like or NRG) to the extracellular lobe of ErbB1, ErbB3 (note inactive kinase, marked by a cross) or ErbB4 induces homodimer formation. When ErbB2 is overexpressed, heterodimers form preferentially. Unlike homodimers, which are either inactive (ErbB3 homodimers) or signal only weakly, ErbB2-containing heterodimers have attributes that prolong and enhance ownstream signaling (green box) and their outputs (yellow box). Apparently, homodimers of ErbB2 are weaker signaling complexes than heterodimers containing ErbB2.

Taken from Yarden Y and Sliwkowski MX; Nature review 2; 2001.

ErbB2-induced mammary tumorigenesis was analyzed by generating several mouse models that express variants of the neu gene (65) (66) (76) (77) (78) under the control of the neu promoter (knock in) (79) or the MMTV promoter. Overall, these models developed breast tumors and lung metastasis with different timing and aggressiveness. One of these models is the MMTV-NeuNT model. In this model a mutated constitutively active form of neu/ErbB2 is expressed under the control of the MMTV promoter. This mutated cDNA (neuT, or neuNT) (80) contains a point mutation that generates an amino acid substitution (Val-Glu) in the transmembrane domain of the protein thus, producing constant ligand-independent dimerization of the receptor (81) and subsequent signaling. MMTV-NeuNT models were initially generated by two different groups (65) (76); in both cases the resulting mice developed multifocal breast tumors and lung metastasis with different latency and aggressiveness, possibly due to the number and site of integration of the transgene

3.4 MMTV-PyMT model

Another gene that has been used to generate tumors in the mammary gland of mice is the Polyoma middle-T gene, which was isolated from the Polyoma virus (66). The Polyoma virus produces tumors when inoculated into young mice. Studies identified three different T (for tumors) antigens responsible for the tumor phenotype: 100 kDa (large or LT antigen), 55 kDa (middle or MT antigen) and 22 kDa (small or ST antigen). Although the three antigens can participate in tumor formation, early studies rapidly associated the middle T antigen (PyMT) with the transformed properties of the Polyoma virus (82).

The PyMT gene encodes a 421 aa long protein. This protein contains in its sequence a predicted transmembrane domain, and an intracellular portion **(Figure 5)**. Immune-cytochemical assays reveal that PyMT is localized in the plasma membrane and inside the cell in close association with intracellular membranes, particularly endoplasmic reticulum. Additionally, PyMT seems to be also in contact with skeleton/cytoskeleton elements (82). In its intracellular portion PyMT contains multiple phosphorylation sites including tyrosine, serine and threonine amino acids **(Figure 5)** (83) (82). Although PyMT has not known endogenous catalytic activity, it interacts with several host proteins acting as scaffold (82) (84). Some of these interactions confer the PyMT-associated tyrosine/threonine kinase activity and promote the transforming potential of PyMT (83) (84).

I will briefly summarize some of the relevant signaling events stimulated by PyMT. Upon interaction with the plasma membrane, PyMT binds the protein phosphatase 2A (PP2A). This protein recruits different (but not all) members of the Src-tyrosine kinase family, such as Src (its major partner in mouse), Yes and Fyn (83) (82). The formation of the PyMT-Src complex increases the tyrosine kinase function of Src (83) (82) (84) (85). PyMT is itself a substrate of Src, therefore, activation of Src increases the phosphorylation of the PyMT Tyr sites Y250, Y315, Y322. Tyrosine phosphorylation activates several signaling pathways that are relevant for cell transformation **(Figure 5)** (82). Some of these pathways will be shortly described. Phosphorylation of Y250 recruits and activates adaptors ShcA family members, which eventually activates the Ras signaling pathway (83) (82). Additionally, PI3K is activated through the interaction with the Y315-P of PyMT and its activation has been associated with prevention of apoptosis and promotion of cell cycle progression. Although it is required, the activation of PI3K is not sufficient for cell transformation (83) (82). Additionally, phosphorylation of Y322 increases phosphorylation of PLCγ1 which seems to have a role in lung metastasis progression (82).

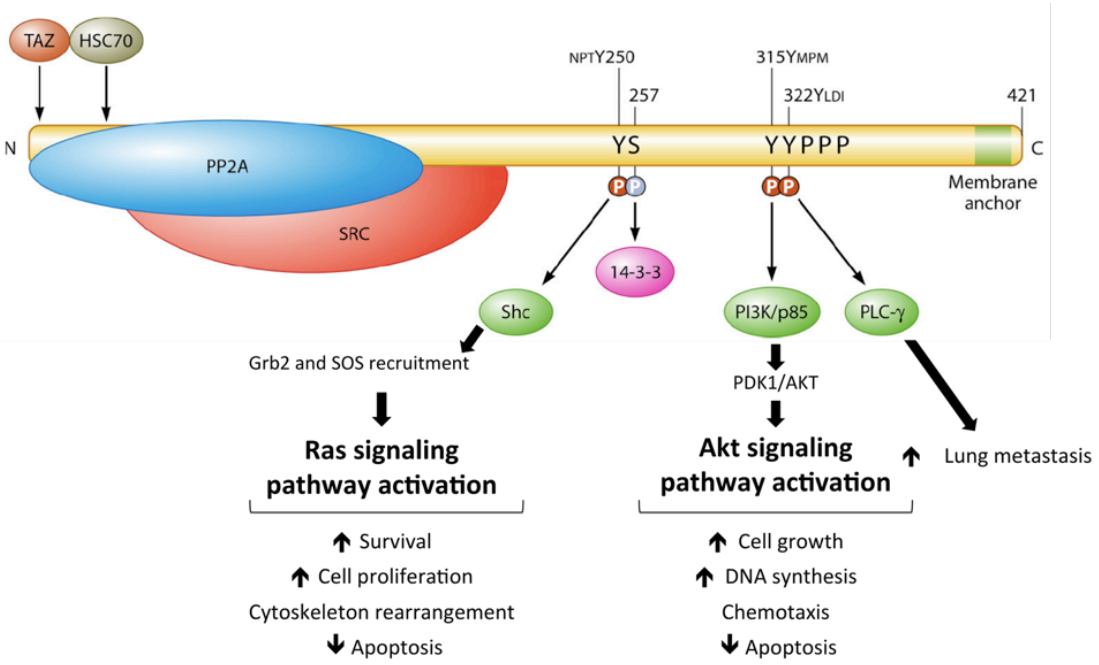


Figure 5. Simplified scheme of PyMT binding proteins and some of the transduced intracellular signaling pathways

Upon interaction with the plasma membrane, PyMT associates with (although does not directly bind to) a member of the Src-tyrosine kinase family, such as Src, Yes or Fyn. This binding results in the phosphorylation of several tyrosines. The binding proteins Shc, PI3K, phosphoinositide 3-kinase, and PLCγ1, activate several intracellular pathways that result in cellular transformation. In addition, PyMT contains serine and threonine phosphorylation sites. The phosphorylation of serine 257 results in the interaction with the 14-3-3 proteins. *PPP represents the proline rich region, which is* important for transformation even if its mutant has a normal tyrosine kinase activity.

Modified from Fluck M. and Schaffhausen B.; Microbiol Mol Biol Rev. 73 (3); 2009.

Furthermore, phosphorylation of PyMT in serine 257 and 283 and in threonine 291 recruits and activates cellular proteins that participate in PyMT-driven cell transformation (83) (82). Additionally, other proteins, such as TAZ and HSC70 family members that bind PyMT in a phosphorylation-independent fashion, can participate in PyMT-driven tumor formation.

Mice that express PyMT under the control of the MMTV promoter develop multifocal breast tumors and lung metastasis. This model, called MMTV-PyMT (PyMT or PyV-mT), has been widely used precisely because mice develop tumors faster than other breast cancer models and because a high proportion of mice develope lung metastasis (86).

4. COMPONENTS OF BREAST TUMORS

Tumors are composed of different cell types including cancer and stromal cells, and an extracellular matrix (ECM). Moreover, the composition of the tumor is not static and changes dramatically through tumor progression.

4.1 Neoplastic epithelial cells

Cancer cells in tumors present multiple differentiation states and genetic modifications. The heterogeneity of tumors is partially explained by clonal evolution and environmental differences (87). This genetic and epigenetic diversification produces subpopulations with distinct and complementary abilities that facilitate tumor growth. Cancer stem cells (CSC) are defined as a subpopulation of cancer cells able to seed new tumors that histologically resemble the original tumor, and to generate daughter cells with proliferative capability but not regenerative potential (88). The cancer stem cell model contains that tumors derive and renew from CSC (87) encouraging the development of new strategies to identify and selectively kill CSC. CSC express at their surface specific combinations of receptors that allow their identification and isolation. In breast cancer the expression of the surface receptors CD24 and CD44 is associated with stem cell properties (89).

4.2 Tumor-associated stroma

The relevance of the tumor microenvironment was highly neglected until the use of mouse models evidenced that normal microenvironments repress tumor formation whereas malignant microenvironments promote it (90). The tumor microenvironment is composed by stromal cells, small molecules as growth factors or cytokines and extracellular matrix.

4.2.1 Stromal cells

Although stromal cells do not accumulate mutations or genomic alterations (91)(92) these tumor-associated cells are not normal but rather represent “activated” versions of stromal cells present in normal tissues. Overall we can classify stromal cells in **(a)** cells of the vascular system like endothelial cells from blood and lymph vessels and pericytes; **(b)** cells of the immune system like T and B lymphocytes, macrophages, mast cells, neutrophils, natural killers, among others; and **(c)** mesenchymal cells like cancer associated fibroblasts (CAF), and adipocytes, which are highly concentrated in the mammary gland.

4.2.2 Extracellular matrix (ECM)

The extracellular matrix is an insoluble, highly organized network of proteins, proteoglycans and glycosaminoglycans, that is used as a 3D scaffold to support cells. There are different kinds of ECM: (a) the basement membrane, which is a thin and tightly crosslinked network (50-100 nm) consisting of

laminins, collagens and other molecules; (b) the interstitial matrix, which is mainly composed by distinct collagens and non-collagen glycoproteins (such as fibronectin, vitronectin, tenascin) and proteoglycans; and (c) specialized ECM which combines characteristics of basement membrane and interstitial matrix (93).

The composition of the ECM in a tumor is not the same as in normal tissues. Specific tumor ECM compositions modify the biophysical characteristics of the tissue and the fate of the cancer cells (94).

5. TENASCIN C

Tenascin-C (TNC) is a major ECM component of the tumor microenvironment. This protein is highly expressed during embryogenesis, but in adult tissues its expression is limited to wound healing, nerve regeneration, tissue involution and to other pathological processes including vascular diseases and cancer (95)(96). TNC belongs to a family of glycoproteins known as the Tenascin family comprising Tenascin R, Tenascin W, Tenascin X, and its charter member TNC (97).

In its primary structure TNC comprises 4 types of structural domains: an N-terminal assembly domain, 14 1/2 epidermal growth factor-like (EGFL) domains, several fibronectin type-III (FNIII) domains and a C-terminal fibrinogen-like domain (**Figure 6A**). The number and organization of FNIII repeats is determined by alternative splicing that results in almost 30 different TNC isoforms (98) with distinct expression patterns and cellular effects. Before secretion, 6 monomers of TNC assemble together to form a hexamer called hexabrachion. In this structure the monomers are held together by their N-terminal assembly-domain whilst the rest of the protein forms the arms of the hexamer. Although TNC is usually organized as hexamer, trimers and nonamers have been also reported (99). The multiple domains of TNC mediate its interaction with other ECM and different cell surface molecules. Therefore TNC triggers signaling in a context-dependent fashion and generates cell-type and organ-specific responses. Although TNC is mainly expressed by stromal cells (100)(101), myoepithelial cells (102) and cancer cells (103) could also be a source of TNC.

5.1 TNC in breast carcinoma

TNC is expressed in the mammary gland during morphogenesis. Its expression is however limited from embryonic day 13 to 16 (in mouse) surrounding the mammary epithelial stalk and briefly during branching, surrounding the newly formed endbuds (100). In the mature mammary gland TNC is not expressed in virgin, pregnant or lactating female mice but reappears during involution (100)(104)(105).

It seems that TNC is absent during the first steps of breast tumor formation such as in hyperproliferative non-neoplastic lesions and in hyperplastic alveolar nodules (100). In DCIS TNC is not or weakly expressed (106). A high percentage of invasive breast carcinomas (more than 70% in most of the reports) express TNC (107)(108)(109)(110). In these tumors TNC is localized around tumor cell nests (107)(111)(112)

preferentially at the invasion border (109), around ducts (107)(112), around vascular channels (112) and in the distal stroma **(Figure 6B)**. Although most of the times expression of TNC co-localizes with stromal-cell rich areas suggesting stromal cells as source of TNC, some reports show TNC expression by cancer cells (113)(111) suggesting that breast cancer cells are also able to produce TNC.

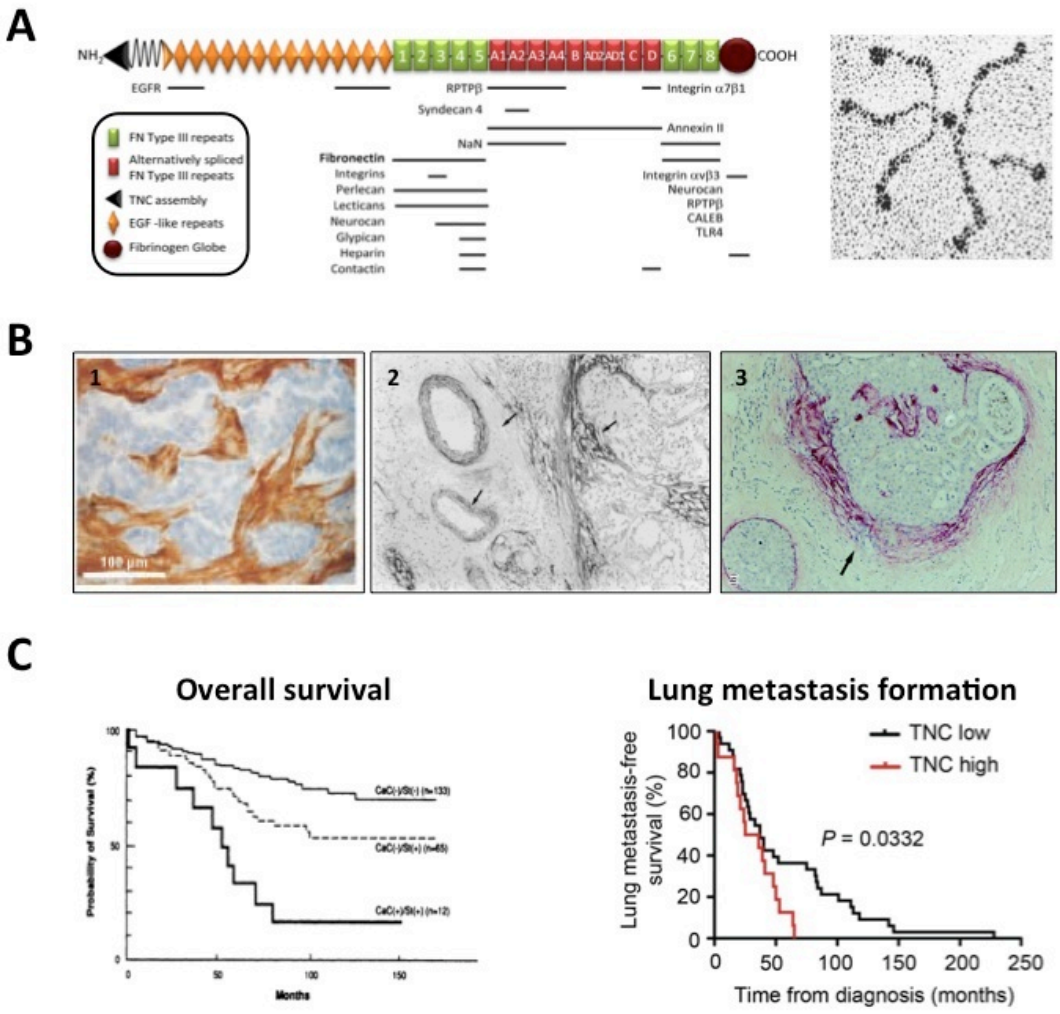


Figure 6. TNC structure and expression in breast cancer

(A) Modular composition of TNC and potential binding partners (left). *Taken from Van Obbergen Schilling et al.; Int J Dev Biol. 55; 2011*. At right it is showen an electron micrograph of TNC hexamer. *Taken from Orend & Chiquet-Ehrismann; Cancer Letters 244; 2006*.

(B) Expression patterns of TNC in breast carcinomas. 1. TNC is in the stroma and form tracks in ductal carcinomas. *Taken from Degen et al., Cancer Research, 67, 2007*. 2. Tenascin expression at the blood vessel walls and stroma (arrows) in the vicinity of the infiltrating tumor. *Taken from Iskaros et al. Journal of Surgical Oncology 68, 1998*. 3. Periductal tenascin-C expression in intraductal carcinomas. Arrow points the microinvasive foci. *Taken from Jahkola et al. European Journal of Cancer, 1998.*

(C) Expression of TNC in breast carcinoma correlates with the overall survival of the patients (left, taken from Ishiara et al. Clinical Cancer Research 1, 1995) and with the lung metastasis-free survival. *Taken from Oskarsson et al., Nature Medicine 17, 2011*.

In view of the high number of breast tumors that express TNC, several studies explored a potential link between TNC levels and other parameters relevant for cancer including prognosis **(Figure 6C)**. Two studies reported that TNC expression inversely correlates with expression of the estrogen receptor (113)(114). The correlation between TNC expression levels and breast tumor grade is not settled. Whereas some reports indicate that high TNC levels correlate with high-grade tumors (111)(114) some others did not find any correlation (115). A direct relationship between total TNC levels and prognosis is also not clear. Some reports showed that patients harboring tumors with high concentration of TNC have lower survival (104) (114)(116); but many others did not corroborate this relationship (109)(111)(117). In those cases, even if the total TNC levels do not correlate with prognosis, other parameters such as the localization, the cell source or the expressed isoform of TNC correlate with a bad prognosis. Jankola et al. (1996) found that if TNC is localized in the invasive border of breast tumors the prognosis of the patient is worse (109). In other studies neither the level of expression nor the localization but, the cellular source of TNC (113) or the TNC isoform (102) was found to be a factor of poor outcome. There is also one report that goes completely to the opposite side claiming that expression of TNC is a marker of good prognosis (108). How to understand these discrepancies? Although most of them could be explained by differences in the patient sampling, it is important to keep in mind that most of these studies evaluated the expression of TNC by immunohistochemical techniques; hence, positivity and intensity of the signal depends on the specificity of the antibody, the ability of the antibody to recognize the expressed TNC isoform(s), and of the quality of the tissue.

Overall the data indicate that a high TNC correlates with malignancy of breast cancer, motivating the study of the role of TNC in tumorigenesis.

5.2 TNC in angiogenesis

Although TNC is not present in normal blood vessels, it is expressed around tumor blood vessels (111)(118), suggesting a role of TNC in angiogenesis. *In vitro* assays demonstrated that EC bind to TNC by different transmembrane proteins including $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins (119) and annexin II (120). Furthermore, TNC appears to modulate the behavior of EC increasing their proliferation, promoting EC migration, and augmenting sprouting and tube formation (99). Whether this is a consequence of a direct binding of EC to TNC is not clear. It is possible that the interaction of cancer cells with TNC increases secretion of pro-angiogenic growth factors like VEGFA and PDGF-BB and endothelin receptor, thus promoting angiogenesis (99).

5.3 TNC in cancer cell adhesion and proliferation

An interesting observation is that TNC expression appears to correlate with tumor size (114) and with the number of proliferative cells in breast tumors (115). One of the first approaches used to analyze the

effect of TNC on cancer cells *in vitro* was to grow cells in the presence of soluble or coated TNC. Using this strategy it was observed that TNC increases breast tumor cell proliferation (121)(122) and three different mechanisms were described.

Huang et al. (2001) tested adhesion and proliferation of the breast cancer cell line MDAMB 435 on a mixed fibronectin (FN) and TNC substratum or on FN alone (121). They reported that cancer cells, seeded on FN/TNC, adhere less and proliferate more than on FN. The authors linked this enhanced proliferation to a mechanism where TNC binds to the FNIII13 domain in FN and thus blocks its interaction with syndecan-4, a co-receptor of integrin $\alpha_5\beta_1$, the major fibronectin adhesion receptor.

Martin D et al. (2003) reported another TNC-depending proliferation mechanism. Using the MCF7 breast cancer cell line they confirmed that TNC increases cell proliferation (122). A comparative screen showed that cells seeded on FN/TNC express more 14-3-3 tau on RNA and protein level than cells seeded on FN. Further analysis with another breast cancer cell line, MDAMB 231, corroborates these data and found that the higher proliferation of the cells is due to 14-3-3 tau regulated degradation of the cell cycle-regulatory protein p21 (123). Interestingly, overexpression of 14-3-3tau in MCF7, an ER positive cell line, produces insensitivity to tamoxifen. Tamoxifen is an estrogen receptor antagonist, widely used in breast cancer therapy. This result might explain why tumors resistant to tamoxifen treatment express higher levels of TNC (117).

Finally Taraseviciute and collaborators (2010) showed that the proliferation promoting effect of TNC correlates with an increased expression of c-met, a gene that encodes the HGF receptor (106). In presence of TNC the non-malignant breast cells MCF-10A formed disorganized three-dimensional acini with a discontinuous basement membrane. This disorganization of the acini correlated with increased cell proliferation and increased c-met expression without any change in apoptosis.

Although growing cancer cells in the presence of coating or soluble TNC provides much relevant information about the effect of TNC on cancer cells, it suffers of several drawbacks. In the first place soluble TNC exhibits different properties than TNC in a 3D context, moreover, TNC produced by cancer cells is different to that produced by normal cells (99). TNC exists in different isoforms inducing different cell responses, hence this strategy tests the effect of only one of the TNC isoforms. Moreover, TNC can be expressed by breast cancer cells themselves (102), while the observed effects described here are a result of the exposition of cells to ectopic and endogenous TNC.

Another way to study the effect of TNC is by blocking its expression in cells of interest *in vitro*. Using knockdown (KD) strategies several groups analyzed proliferation, migration, and the tumorigenic and metastatic potential of TNCKD breast cancer cells. Surprisingly KD of TNC did not modify proliferation in cells growing in 2D but it did when cells were grown as spheres. These results suggest that TNC could play a role in tumor initiation, presumably by promoting stemness characteristics of tumor cells, and it was demonstrated that this is important during lung metastasis formation (41)(124)(125).

5.4 TNC promotes breast cancer cell migration, invasion and EMT

In several breast cancer cells TNC has been shown to promote migration and invasion *in vitro*, either by knockdown approaches, the use of TNC blocking antibodies or adding exogenous TNC. In MDAMB 231 cells TNC mediated invasion could be blocked by MMP-inhibitors (126). There are also several evidences that TNC promotes EMT. In breast cancer TNC expression correlates with the expression of Vimentin (127). Upon combined TGFβ-1 and TNC treatment MCF-7 and T47-D breast cancer cells lost expression of membranous E-cadherin and β-catenin, which is indicative of an EMT-like phenotype (128). Also in another study TNC has been demonstrated to promote partial EMT in MCF-7 cells. Upon growth on a TNC substratum those cells changed from an epithelial to a more fibroblastic phenotype (122).

Other cellular processes that were affected in TNCKD cells are migration and invasion (41)(124). The presence of TNC at the invasion border and the invasion into lymph nodes (114) correlates with metastasis to distant tissues (109). It seems that TNC increases the invasion of breast cancer cells by promoting EMT (128).

5.5 TNC in metastasis

TNC has been observed in the invasive border of breast tumors (115) and its expression has been correlated with invasion and /or metastasis (99). Interestingly, TNC has been predicted in three analysis on a large cohort of human breast tumors as part of lung metastasis signature (129) (130) (124). In order to identify genes responsible for breast cancer cells metastasizing specifically to the lung, Minn and co-workers used MDAMB231 breast cancer cells selected to home to the lung. TNC was included in a set of genes, when expressed in the primary tumor, mediate breast cancer metastasis to the lung (129). Likewise Calvo et al. (2005) determined genes that promote lung macrometastasis formation in a MMTV-Myc/VEGFA induced breast tumors also found TNC as part of the lung metastasis signature, now in an immunocompetent mouse model (41). Subsequent research demonstrated that overexpression of TNC in cancer cells could occur due to the down regulation of miR-335, a microRNA that targets TNC and that is decreased in metastatic breast cancer cells. Interestingly, down regulation of miR-335 did not modify proliferation of cancer cells or the size of the primary tumor, but it reduced migration of the cells in *in vitro* assays and the capability of cancer cells to form metastasis (130).

With the purpose of elucidate the role of TNC promoting breast cancer lung metastasis. Tavazoie and collaborators (2008) tested the lung metastasis colonization capability of the breast cancer cell line MDAMB231 with or without expression of TNC when cells were tail vein injected in immunodeficient mice. They observed that in the absence of TNC this cell line reduce its ability to colonialize the lung (130). Later, Oskarsson and collaborators (2011) confirmed these data injecting MDAMB231 LM2 or CM34-M1 cell lines intravenously (tail vein) or into the mammary fat pad of immunedeficient mice and also observing that the absence of TNC correlate with a decrease in lung metastasis colonization (124). By

using the MDAMB435 cell line wild type and TNC knock down Calvo and collaborators (2008) injected in the mammary fat pad of immune compromised mice also confirming that cancer cells that do not express TNC form less metastasis in the lung (41). Finally, O'Connell and collaborators (2011) injected cells 4T1 intravenously and into the mammary fat pad of immune competent Balb/c mice TNC wild type or TNCKO. They observed that when stromal cells are not able to produce TNC the ability of the 4T1 cells to colonialize the lungs decrease (101). Altogether these studies suggest that TNC expressed either by cancer cells or by stromal cells promotes lung metastasis colonization.

Contrary with the results obtained in cell grafting studies are the results obtained by Talts and collaborators (1999). They crossed mice that are not able to express TNC (TNCKO) with MMTV-PyMT mice that spontaneously develop breast tumors and metastasis to lung. Although they observed that in the absence of TNC the organization of the tumors is different and they are more infiltrated by macrophages; surprisingly, the lack of TNC does affect neither primary tumor growth parameters (latency, number of tumors and size) nor lung metastasis colonization (131).

With the goal to understand the molecular mechanisms driven by TNC, Oskarsson and collaborators (2011) analyzed the ability of cancer cells TNCwt and TNCKD to growth as spheres *in vitro*, a method to select cells with tumor- initiating capabilities *in vitro*. Although in this assay TNC produced by cancer cells did not modify the expression of known breast cancer stem cell markers such as CD44 and CD24, it promoted the expression of components of the Notch and Wnt signaling pathways (Msi1 and Lgr5, respectively) that are crucial for stem and progenitor cell growth and/or survival. Indeed, the specific knockdown of these downstream components provided compelling evidence as it decreased lung metastasis formation (124). Exploring the kinetics of lung metastasis formation, the authors further nicely demonstrated that expression of TNC by cancer cells is crucial during the early steps of lung metastasic colonization while its knockdown after tumor cells could settle did not affect lung metastasis progression (124). Presumably stromal cells that are recruited by cancer cells in the metastatic niche may secrete TNC which could promote cell survival (124)(101).

Altogether these studies support the hypothesis that TNC have an important role in lung metastasis colonization.

TNC is not the only ECM protein unambiguously involved in metastatic colonization (132) (133). Indeed other examples of ECM molecules that may be important include fibronectin which was described as expressed in pre-metastatic niches (43) and SPARC which was also identified as part of the lung metastatic signature (129). Finally it is interesting to note that periostin, a well-known direct binding

partner of TNC in ECM was recently identified as a major component of the metastatic-initiating cell niche in the MMTV-PyMT murine model of breast cancer (132) (134).

Globally, data from breast cancer patients, *in vitro* assays and murine breast cancer models suggest that TNC promotes breast cancer progression. To gain novel insights, my thesis aimed at using murine models of breast cancer to better understand the several contributions of TNC to breast cancer progression *in vivo*.

OBJECTIVES

The main goal of my thesis is to try to comprehensively understand the several possible contributions of TNC during breast cancer establishment and progression to metastatic disease. My project uses two different stochastic oncomouse models, the “PyMT” (MMTV-PyMT) and the “NeuNT” (MMTV-NeuNT) oncogene-induced models of breast cancer, in which females develop multifocal ductal adenocarcinoma of the breast that ultimately metastasize to the lung.

The specific aims of my thesis are:

Objective 1: Analyze the effects of TNC overexpression on PyMT and NeuNT breast cancer progression.

Objective 2: Analyze the effects of TNC loss on PyMT and NeuNT on breast cancer progression.

Objective 3: To develop syngenic breast cancer cell lines from PyMT and NeuNT mouse models.

MATERIAL AND METHODS

1. Mouse handling and *in vivo* experiments

All mice were housed and handled according to the guidelines of INSERM (Directive 2010/63/EU on the protection of animals used for scientific purposes). During this work two different breast cancer mouse models were used: MMTV-NeuNT and MMTV-PyMT (both in FVB/NCrl genetic background), which were generously donated by Gerhard Christofori (University of Basel, Switzerland) originally created by William Muller, Montreal University (Canada).

1.1 MMTV-TNC model

Martial Kammerer had generated the MMTV-TNC model in the host laboratory during his postdoctoral stay. Briefly, the murine DNA sequence of TNC was obtained from the pBluescript SK (+) blackbone (gift from Dr M. Kusakabe, Riken, Japan). This sequence corresponds to the BAA14355 and CAD834047 entries in NCBI. It contains a part of the 5' untranslated region (5'-UTR), the murine TNC coding sequence and its 3'-UTR region without the polyadenylation signal. In the 5' region of TNC a chimeric intron was added to stabilize the generated transcript *in vivo*. This sequence was cloned in the MMTV-IRES-Luciferace vector, generously donated by A. Fantozzi from the G. Christofori team (University of Basel, Switzerland) (**Figure 7A**). *In vitro* characterization of the construct was done using MCF-7 cells and the expression of the transgene was confirmed by reverse transcription quantitative polymerase chain reaction (RT-qPCR), immunofluorescence microscopy and luciferase assays.

Establishment of the transgenic lines was done in collaboration with Daniela Klewe-Nebenius at the Biozentrum in Basel, Switzerland. Several injections were done into oocytes from either C57BL/6 or FVB/NCrl mice. Successful insertion of the full transgene was analyzed by PCR (**Figure 7B**) and the founder lines were transferred to our animal facility.

During my thesis work, only MMTV-mTNC mice in the FVB/NCrl background were used.

The expression of the transgene (RNA) in the mammary of virgin female MMTV-mTNC mice was verified by RT-qPCR (**Figure 7C**).

To obtain double transgenic mice (PyMT-mTNC and NeuNT-mTNC) heterozygous oncogenic males were crossed with heterozygous MMTV-mTNC females.

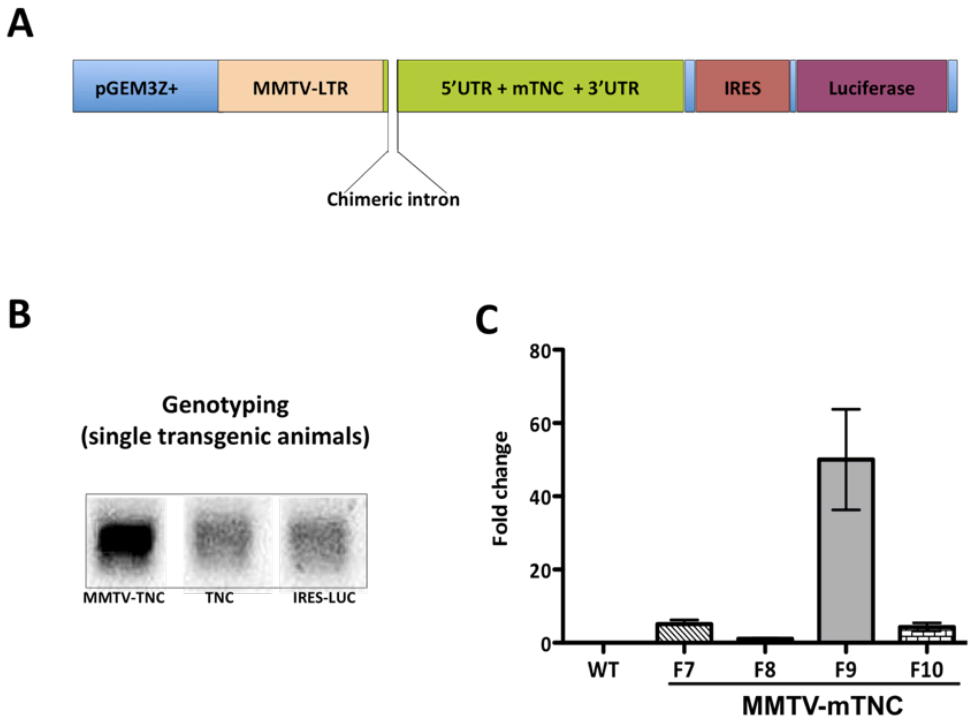


Figure 7. MMTV-mTNC model

(A) Schematic representation of the MMTV-mTNC plasmid containing mTNC-IRES-Luciferase sequences under the control of the MMTV promoter.

(B) Transmission of the transgene was verified by PCR using primers against different regions of the transgene (MMTV-TNC boundary, TNC gene and IRES-luc boundary).

(C) Mammary glands of control (wt) and MMTV-mTNC mice from founders 7, 8, 9 and 10 (F7 n = 5; F8, n = 3; F9, n = 5; F10, n = 5) of virgin mice between 8 and 12 weeks old were recovered and the expression of the transgene mRNA was quantified by RT-qPCR.

1.2 TNC knock out mouse model

TNC +/- mice in 129/Sv genetic background were generously donated by Reinhard Fässler. Ten consecutive crosses with FVB/NCrl mice (Charles River) were done to obtain TNC +/- mice in an almost pure FVB genetic background.

Oncogenic TNCKO mice (PyMT-TNCKO and NeuNT-TNCKO) were obtained by using two different breeding protocols: 1) TNC +/- oncogenic males were crossed with TNC +/- females to obtain oncogenic TNC +/+, TNC +/- and TNC -/- littermates. 2) Oncogenic TNC +/+ or TNC -/- male were crossed respectively with TNC +/+ or TNC -/- female to obtain heterozygous oncogenic mice TNC +/+ and TNC -/- from different breedings. The absence of TNC in PyMT and NeuNT TNC -/- breast tumors was confirmed by Western blot **(Figure 8A and B).**

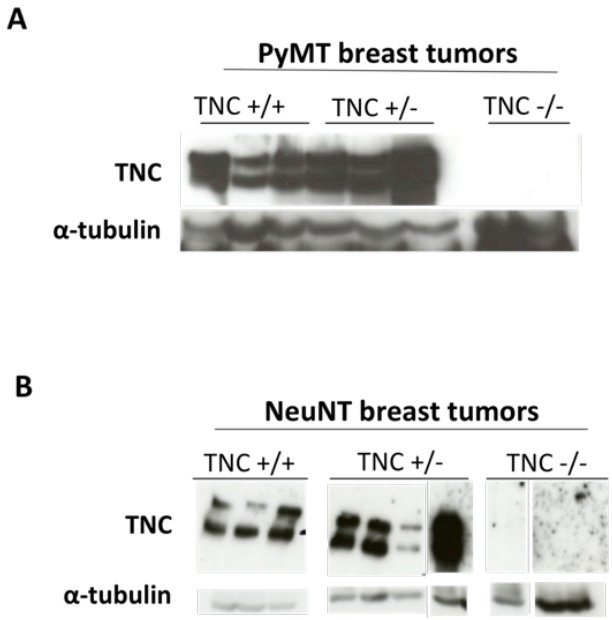


Figure 8. TNC expression in breast tumors

(A) Western blot of breast tumor lysates from PyMT, TNC +/+ (n = 3), TNC +/- (n = 3) and TNC -/- (n = 2) of 110 -121 days of age female mice . Alpha tubulin was used as loading control.

(B) Western blot of breast tumor lysates from NeuNT, TNC +/+ (n = 3), TNC +/- (n = 4) and TNC -/- (n = 3) of 3 months bearing tumor mice. Alpha tubulin was used as loading control.

The tumor latency in oncogenic mice was determined by examining the mammary glands of the female by palpation twice weekly. Palpations were done in blind conditions with respect to the genotype of the mice. Total tumor weight per mouse (tumor burden) was determined during dissection.

To determine the genotype of mice, DNA was extracted from tail biopsies using directPCR lysis reagent (102T, Viagen Biotech) and was amplified by PCR using specific primers (**Table 1**). The genotype was determined twice: once after birth and after dissection of experimental animals.

Table 1. Primers used for genotyping

		Forward	Reverse
GAPDH		5'-ACCACAGTCCATGCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA-3'
MMTV-mTNC	***MMTV-mTNC***	5'-CCGCTCGTCACTTATCCTTC-3'	5'-CTGTAGGGTGCCTGAACTCC-3'
	ectopicTNC	5'-CTGGTGCACCCAGAGACTTT-3'	5'-TCTCGACAAGCCCAGTTTCT-3'
	IRES-Luc	5'-AATGGCTCTCCTCAAGCGTA-3'	5'-AACAGTACCGGAATGCCAAG-3'
NeuNT	***neuNT_1***	5'-GGAAGTACCCGGATGAGGAGGGCATATG-3'	5'-CCGGGCAGCCAGGTCCCTGTGTACAAGCCG-3'
	neuNT_2	5'-TCCTGTGTGGATCTGGATGA-3'	5'-GTCAGCGGCTCCACTAACTC-3'
PyMT		5'-CGGCGGAGCGAGGAACTGAGGAGAG-3'	5'-CAGAAGACTCGGCAGTCTTAGGCG-3'
TNCKO	***wt***	5'-CTGCCAGGCATCTTT-3'	5'-TTCTGCAGGTTGGAGGCA-3'
	TNCKO	5'-TTCTGCAGGTTGGAGGCA-3'	5'-CTGCTCTTTACTGAAGGCTC-3'

1.3 Syngeneic model

A suspension of NeuNT193 (NT193) or PyMT248 (PT248) breast cancer cells (see below) was injected orthotopically (into the mammary gland) or heterotopically (subcutaneously) in FVB/NCrl immune competent female mice (Charles River, 60 days of age for NT193 and 120 days of age for PT248). Cells were injected in both flanks of mice.

To realize the orthotopic injections mice were anesthetized, the skin was incised and the 4th mammary glands were exposed. Ten million cells (in 100 microliters of PBS 1X) were injected with a 25 gauge needle per injection, and two contralateral injections were done for each mouse. Afterwards, the skin was aligned and stapled with steel surgical clips. Tumor size was measured once weekly using a caliper. Mice were euthanized at different time points after cell injection (1, 3 and 5 months) and breast tumors and lungs were processed for histological analysis.

Determination of direct lung metastagenicity was done by injecting one million cells (in 100 microliters of PBS 1X) in the tail vein. Mice were sacrificed at different time points after cell injection (2 after one month; 6 after 5 months) and lung tissue was processed for histological analysis.

2. Mammary gland whole mount preparation and analysis

Mammary glands of NeuNT TNC +/+ and TNC -/- mice were dissected at 4.5 months of age before palpable tumors were detected **(Figure 9)**. The right 4th mouse mammary gland was excised, spread onto a microscope slide, and fixed overnight in Carnoys solution (75% ethanol, 25% glacial acetic acid). Next day slides were soaked 15 min in ethanol 75%, gradually hydrated and soaked in carmine alum staining solution (0.2% carmine dye, 0.5% aluminum potassium sulfate) overnight at room temperature (RT). Slides were destained (2 h in 70% ethanol, 2% hydrochloric acid), dehydrated, delipidated in toluene for several days and mounted.

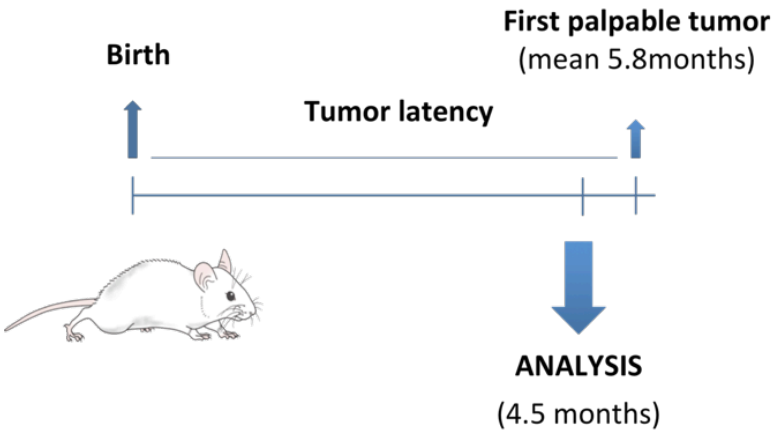


Figure 9. Scheme representing the time point when tissue was collected for early tumor lesions analyses

Mammary glands of 4.5 months old mice were dissected. At this time point no tumor is detected by palpation.

Mammary gland whole mounts were analyzed by using an Olympus SZX7 stereomicroscope and the Axio Imager A1 microscope (Carls Zeiss). Since normal mammary glands in age-matched female virgin mice harbored nodules up to 200 μm in diameter we considered only nodules bigger than 200 μm as early tumor lesions. The size of the nodules was determined by using the AxioVision 4 software.

3. Histological and immunohistological analysis

3.1 Tissue embedding into paraffin

After dissection tissue from mammary glands, breast tumors and lungs was fixed with PFA 4% overnight at 4°C, washed, dehydrated and embedded in paraffin. Mammary glands and breast tumors were embedded in paraffin blocks and cut in 7 μm thick sections. For histological analysis slides were dewaxed and stained with hematoxyline (01562E, Surgipath) for 5 min at RT. Dye excess was removed under tap water and the slides were immersed into a differentiating solution (1% HCl in absolute ethanol, for 7 seconds), followed by washing under tap water for 10 min. Afterwards, slides were stained with eosine (31273-7, RAL) for 10 seconds at RT, the excess of dye was washed under tap water and slides were dehydrated and mounted. The histological analysis of breast tumors was done by the pathologist Marie-Pierre Chenard-Neu (Department of Pathology of the Hautepierre Hospital in Strasbourg, France).

For an overall quantification of lung metastasis the biggest lung lobe was fixed, cut in about 3 mm thick sections and embedded in paraffin blocks. Tissue blocks were cut in 7 μm thick sections, dewaxed and stained with Giemsa (320310-0125, RAL) for 2 hours at 37°C. Sections were further processed in a 0.5% aqueous acetic acid solution, dehydrated and mounted with Eukitt.

For immunohistochemistry, paraffin embedded tissue was re-hydrated and the antigens were unmasked by boiling in 10 mM pH 6 citrate solution for 20 min. Cooled slides were washed and incubated in a peroxide solution (0.6% H_2O_2, 0.1% triton X-100 in PBS) to eliminate endogenous peroxidase activity. Non-specific binding sites were blocked with a blocking solution (5% normal goat serum in PBS) for 1 hour at RT and then avidin/biotin receptors were blocked by using the avidin/biotin blocking kit as recommended by the manufacturer (Vector). Slides were incubated with the first antibody (Table 2) overnight at 4°C in a humidified container. Next day, slides were washed and incubated for 45 min at room temperature with a secondary antibody (coupled to biotin). The detection of peroxidase was done using the Elite ABC system (VECTASTAIN) with DAB as substrate. Finally tissue was stained with hematoxylin, dehydrated and mounted.

3.2 Preparation of fresh-frozen tissue and analysis

After dissection mammary glands and breast tumors were embedded in OCT (Tissue-Tek® OCT™ Compound, Sakura) and snap frozen in cooled isopentane liquid nitrogen. For improved preservation of lung tissue architecture OCT was directly injected into the trachea of sacrificed mice. The two medium-sized lobes were snap frozen and processed as described for breast tumors and mammary glands (see above).

Lung tissue was cut into 7 µm thick sections placed on SuperFrost Plus slides (Thermo Scientific). Slides were stored at -20°C until use. For histological analysis slides were fixed with 4% PFA for 20 min at RT and stained with hematoxylin-eosin as described above for paraffin-embedded tissue.

Immunohistochemistry on fresh frozen tissue was done using fluorescent-labeled secondary antibodies (Immunofluorescent protocol, IF). Briefly, tissue was air-dried and unspecific signal was blocked with blocking solution (5% normal goat serum in PBS) for 1 hour at RT. Tissue sections were incubated with the primary antibody (Table 2) overnight at 4°C in a humidified container. The following day the primary antibody was removed and the tissue was incubated with a fluorescent secondary antibody for 1 hour at RT. Slides were washed and incubated with a DAPI solution to visualize the nuclei of the cells (5 min at RT). Excess of the dye was removed and tissue was mounted with FluorSave™ Reagent (345789, Calbiochem). Fluorescent signals were analyzed with a Zeiss Apotome microscope. In all the IF presented, the nuclei are visualized by DAPI stain (blue).

Table 2. Antibodies used for immunohistological analyses

Antigen	Protocol	Reference	Working dilution /concentration	Source
mTNC	IF	mTN12 (135)	4 µg/ml	rat
HER2/ErbB2	IF	Cell signaling #2242	1:50	rabbit
	IHC	Thermo Scientific (clone e2-4001 + 3B5)	0.5 µg/ml	mouse
PyMT	IHC	Santa Cruz: sc-53481	1:500	rat
Keratin 8 and 18	IF	Progen GP11	1:500	guinea pig
CD31	IF	e-bioscience	1:100	rat
CD41	IF	Abcam (ab33661)	1:50	rat
S100A4	IF	(136)	1:200	rabbit
Fibronectin	IF	Fibronectin 2.2 (home made)	1 : 200	rabbit
Total Laminin	IF	Dr P Simon-Assmann: LN6.7S	1:5000	rabbit
Ki67	IHC, IF	Thermo Scientific (clone SP6)	1:200	rabbit
Cleaved Caspase3	IHC, IF	Cell signaling #9661	1:600	rabbit

4. Gene expression analysis of tissue

The two medium-sized lung lobes and small pieces of breast tumors were frozen in liquid nitrogen and were kept at -80°C until use. RNA and protein extraction was done using the total RNA and protein isolation kit (Macherey-Nagel) as recommended by the manufacturer.

4.1 RNA purification and RT-qPCR analysis

Total RNA purified from breast tumors and lungs was eluted in 60μl of milliQ water and the final concentration was quantified by measuring the optical density OD 260 nm with a Nanodrop. If the 230/260 ratio was >1.5 RNA was precipitated with 3 M of acetate sodium pH 5 and 3 volumes of absolute EtOH for 1-2 hours at -80°C. The RNA pellet was washed and finally resuspended in 20 μl milliQ water and reanalyzed with the Nanodrop.

For reverse transcription followed by quantitative polymerase chain reaction analysis (RT-qPCR) one microgram of RNA per sample was treated with deoxyribonuclease I, amplification grade (Invitrogen). Afterwards, the RNA was reverse transcribed using high-capacity cDNA reverse transcription kit (Applied Biosystems) and controls were prepared without addition of Reversed transcriptase. Quantitative polymerase chain reaction (qPCR) was done on a 7500 Real Time PCR system (Applied Biosystems) using the Power SYBR® Green PCR Master Mix (Applied Biosystems) and specific primers (Table 3). To quantify relative gene expression levels, the $2^{-\Delta\Delta Ct}$ method was used (137). Cycle threshold values (Ct) from the gene of interest were normalized with respect to the housekeeping gene RPL19 to obtain a ΔCt value. Relative gene expression was then determined by subtracting the mean ΔCt of the control group from the ΔCt of the gene of interest:

$\Delta\Delta Ct = \Delta C_t$[gene of interest] - ΔC_t[mean of the control group]

Determination of the fold change was done using the formula: Fold change = $2^{-\Delta\Delta Ct}$

Table 3. Primers used for RT-qPCR

Gene	Forward primer	Reverse primer
RPL19	5'- ACCCTGGCCCGACGG -3'	5'- TACCCTTCCTCTTCCCTATGCC-3'
Neu	5'- TGGACGGCCTCTGGATTA-3'	5'- CATGATACCCACTGGACCCTA-3'
murine *TNC*	5'- GCGCAGACACACACCCTAGC-3'	5'- TTTCCAGGTCGGGAAAAGCA-3'
ectopic murine *TNC*	5'- AGATTGGGGACATCCAACTG-3'	5'- TCTAGATCTGAGTGGACACCTGCC-3'
Luciferase	5'CTTGGCATTCCGGTACTGTT-3'	5'GCCTTATGCAGTTGCTCTCC-3
Total TNC	5'- GTTTGGAGACCGCAGAGAAGAA -3'	5'-TGTCCCCATATCTGCCCATCA -3'

4.2 Gene expression analysis by RNA profiling

RNA was isolated from primary breast tumor fragments snap frozen with liquid nitrogen using the total RNA isolation kit (Macherey-Nagel) as recommended by the manufacturer. RNA (3 mg per tumor) was treated with RNAse-free deoxyribonuclease I, amplification grade (Invitrogen). Final RNA concentration was determined by

measuring OD 260nm with the Nanodrop. Three breast tumor samples (from three different mice) per genotype (TNC +/+ and TNC-/-) were prepared. Pooled samples were transferred to the IGBMC microarray platform for quality control and validation (Agilent Bioanalyzer), cRNA synthesis and hybridization to Affymetrix Mouse Gene 1.0 arrays following manufacturer's instructions. Raw data were normalized using the RMA (Robust Multi-chip Averaging) method with the Expression Console (Affymetrix).

4.3 Analysis of protein expression levels

Upon RNA isolation, the protein fraction was recovered by precipitation with the Protein Precipitator buffer (Macherey-Nagel) and washed as recommended by the manufacturer. After precipitation the dried pellets were resuspended in lysis buffer (50 mM phosphate buffer, 300 mM NaCl, 1% triton, 8 M urea, 10 mM β-mercaptoethanol and protease inhibitor P8340, Sigma), and were homogenized passing several times through needles of different gauges (21, 23 and 26), centrifuged (15 min at 14000 x g) at RT and stored at -20°C until use.

Protein concentration was determined by the Bradford assay using the Protein Assay Reagent (500-006, Bio-Rad). Total proteins lysates (30 μg) were resolved in a 6% PAGE/SDS gel and subsequently transferred on a PVDF membrane with a pore size of 0.45 μm (IPVH00010, Immobilon). Protein transfer was verified using the Ponceau-S dye (81462, Sigma). To prevent unspecific antibody binding the membrane was soaked for 20 min at RT under shaking in a blocking solution, which contains 5% of Blocking-Grade Blocker (170-6404, Bio-Rad) in PBS/1% Tween. Primary and secondary antibodies (HRP-coupled) were diluted at different concentrations (**Table 4**) in 1.5% of Blocking-Grade Blocker in PBS1X/1% Tween. The membrane was incubated with the primary antibody over-night at 4°C. After extensive washing with PBS1X/1% Tween (3 times 5 min) the membrane was incubated with the secondary antibody for 45 min at RT. After 3 washes of 5 min with PBS1X/1% Tween, immunocomplexes were revealed by addition of detection reagents (RPN2132 or RPN2106, GE healthcare) and the emitted signal was captured in an Amersham hyperfilm ECL (28906837, GE Healthcare).

Table 4. Antibodies used for Western blot

Target	Ref	Dilution /concentration	Host
mTNC	mTN12, (135)	4 μg/ml	Rat
Alpha-tubulin	Oncogene Cat#CP06	1:500	Mouse
Vinculin	Sigma: Clone hVIN	1:400	Mouse
anti-Rat-HRP	GE NA935V	1:5000	Goat
anti-mouse-HRP	NXA931	1:5000	Sheep

5. Gene expression and protein analysis of cultured cells

Cells were seeded in a 6-well plate (2 X 10^5 cells per well) and cultivated for two days at 37°C in a humidified atmosphere (5% CO_2). Cells were lysed with TRI reagent (TR118, MRC) and total RNA isolated as recommended by

the manufacturer. RNA quantification, reverse transcription and quantitative PCR analysis were done as described above. For protein analysis cells were washed with PBS 1X and lysed with Laemmli solution (60 mM Tris-Cl pH 6.8, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue). Cell homogenate was boiled for 10 min just before loading in a SDS-polyacrylamide gel. Western blot analysis was done as described above.

Expression and distribution of candidate proteins were analyzed by immunocytochemistry. Therefore, cells were seeded onto untreated coverslips and cultivated at 37°C in a humidified atmosphere of 5% CO_2. Cells were fixed for 15 min at RT with a 1:1 mix of 4% PFA and cell medium. This mix was removed and a solution of 4% PFA was added to the cells for 15 min at RT. After fixation coverslips were washed and the cells were permeabilized for 10 min at RT with a solution of 0.1% of Triton X-100. This solution was removed and coverslips were washed. The following steps were done as described previously for fluorescent immunohistochemistry on tissue.

6. Establishment of cell lines derived from breast tumors and lung metastasis

Tissue from breast tumors of MMTV-NeuNT or MMTV-PyMT mice were recovered, minced finely using scissors and digested enzymatically using trypsin 0.25% in Versene solution. The cell suspension was passed through a 40 μm nylon filter (BD Falcon) to remove debris and cells were cultured in DMEM medium with 4.5 g/L glucose (41966-029, GIBCO) supplemented with 10% of inactivated fetal calf serum, penicillin (10 000 U/ml) and streptomycin (10 mg/ml). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

The morphology of the cells was analyzed continuously. During the first passages a mixed population of cells with epithelial and mesenchymal morphologies was observed. To remove mesenchymal cells (presumably CAFs) repeated selective trypsinization steps were performed. 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 1X). Detached cells were removed, still adherent (epithelial) cells were then washed with PBS 1X once and the medium was re-freshed. After several passages, cells forming an epithelial cobblestone monolayer were almost pure and found to all express the luminal epithelial cell marker cytokeratin 8/18 and the respective tumor cell specific oncoproteins PyMT or ErbB2. The establishment and characterization of these murine syngeneic tumor cell lines and their derivatives is summarized in **Figure 10** and further detailed in the results section (PyMT: **Figure 28**; NeuNT: **Figures 29-30**). Cell lines from lung metastases ("LM") were established similarly, except that collagenase was added during the enzymatic digestion of tissue.

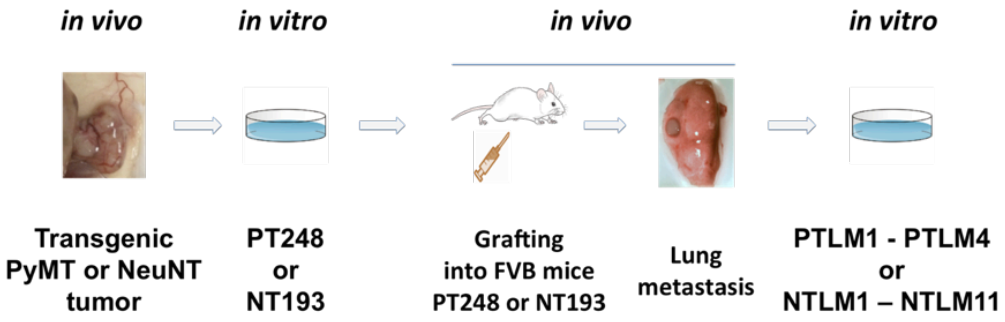


Figure 10. Experimental protocol to syngeneic tumor cell lines and derivatives

Scheme representing the different steps to esatblish the NT193 and PT248 cell lines and their derivatives from leung metastases (PTLM1 to PTLM4 and NTLM1 to NTLM11).

7. TNC knockdown (TNCKD)

The silencing of TNC in mouse cells was done by short hairpin (sh) mediated gene expression knock down (KD). Therefore, lentiviral particles shRNA vectors (Sigma-Aldrich) encoding specific shRNAs for the knock down of TNC were used (**Table 5**). Lentiviral particles encoding a non-targeting shRNA vector were used as a control (SHC202V, Sigma-Aldrich). Transduced cells were selected with normal medium supplemented with 10 µg/ml puromycin and the selection pressure was kept in all *in vitro* experiments.

Table 5. TRC numbers and shRNA sequences

NAME	CLONE ID	SEQUENCE
sh1	TRCN0000312137	CCGGCCCGGAACTGAATATGGGATTCTCGAGAATCCCATATTCAGTTCCGGGTTTTTG
sh2	TRCN0000312138	CCGGGCATCAACACAACCAGTCTAACTCGAGTTAGACTGGTTGTGTTGATGCTTTTTG
sh3	TRCN0000313073	CCGGTCGAAACATGGGAGATCATTTCTCGAGAAATGATCTCCCATGTTTCGATTTTTG
sh4	TRCN0000349824	CCGGGGGTCACTGAGTACCTCATTACTCGAGTAATGAGGTACTCAGTGACCCTTTTTG
sh5	TRCN0000349876	CCGGCAGGCGTAAGCGGGCATAAATCTCGAGATTTATGCCCGCTTACGCCTGTTTTTG

8. Statistical analyses

The GraphPad Prism software (version 5) was used for graphical representations of data and statistical analyses to assess significance of observed differences. All parametric (unpaired Student t test) and non-parametric tests (Mann-Whitney, Kruskall Wallis) were performed in a two-tailed fashion. A Kaplan-Meier survival analysis was performed to analyze tumor latency data, and the log-rank test was used. A contingency analysis was used (a) to compare the numbers of intravascular emboli and parenchymal metastases in NeuNT TNC +/+ and TNC -/- mice (represented as proportions, cf **Figure19F**), Fisher's exact test was used, and (b) to compare the proportion of the

number of early tumor lesions per mammary gland of NeuNT TNC +/+ and TNC -/- mice, Chi-square test was used. Differences were considered significant when p-values were less than 0.05.

RESULTS

Although important insight into the underlying molecular mechanisms has been obtained (124) a comprehensive view about the roles of TNC in breast cancer onset and progression is missing. Therefore, I had studied the roles of TNC in breast cancer by using two different transgenic breast cancer mouse models, the MMTV-PyMT and the MMTV-NeuNT models (thereafter referred as to "PyMT" and "NeuNT", respecitvely). As mentioned before, both models develop invasive adenocarcinomas metastasizing to the lung. Whereas MMTV-PyMT mice develop palpable tumors as early as 4-8 weeks of age and lung metastasis in 85-100% of the cases (138), tumor latency in MMTV-ErbB2 mice reaches 5 to 10 months with a high propensity to form lung metastasis (63) (65) (139) (76). Using these models, I addressed how different levels of TNC affect breast tumor onset and progression. Although a similar analysis was done for both models, the results will be presented separately for an easier understanding.

1. IMPACT OF DIFFERENT TNC EXPRESSION LEVELS ON MMTV-PyMT BREAST TUMORIGENESIS

1.1 Description of TNC expression in PyMT breast tumors and lung metastasis

To validate the use of the PyMT mouse model I first analyzed TNC expression in breast tumors and their derived lung metastases (**Figure 11**). By IF I observed that TNC is expressed in PyMT primary tumors (**Figure 11A-C**). TNC is localized in different areas: within the tumor, TNC is expressed within stromal tracks (**Figure 11A**) and potential blood vessels (**Figure 11B**) suggesting a potential role of TNC in angiogenesis in this model, but also in the stromal area directly surrounding the tumor (**Figure 11C**). Co-staining with total laminin, revealed that both proteins can co-localize; however not always perfectly. TNC is also present in PyMT lung metastases (**Figure 11D-F**). Within metastases TNC forms stromal tracks and in some cases it is also present at the border of the metastasis (**Figure 11D, 11E**) recapitulating the expression pattern observed in primary tumors. A track-like expression of TNC has been previously described in human metastases and in xenograft models of breast cancer (124). In addition I observed high TNC around small clusters of micrometastatic tumor cells, suggesting that TNC might be expressed by stromal cells during early steps of lung colonization (**Figure 11F**). Overall these results indicate that TNC is expressed in PyMT breast tumors and lung metastases and that its expression pattern is very analogous to human breast cancer. Thus this model could be suitable to address the roles of TNC in breast cancer.

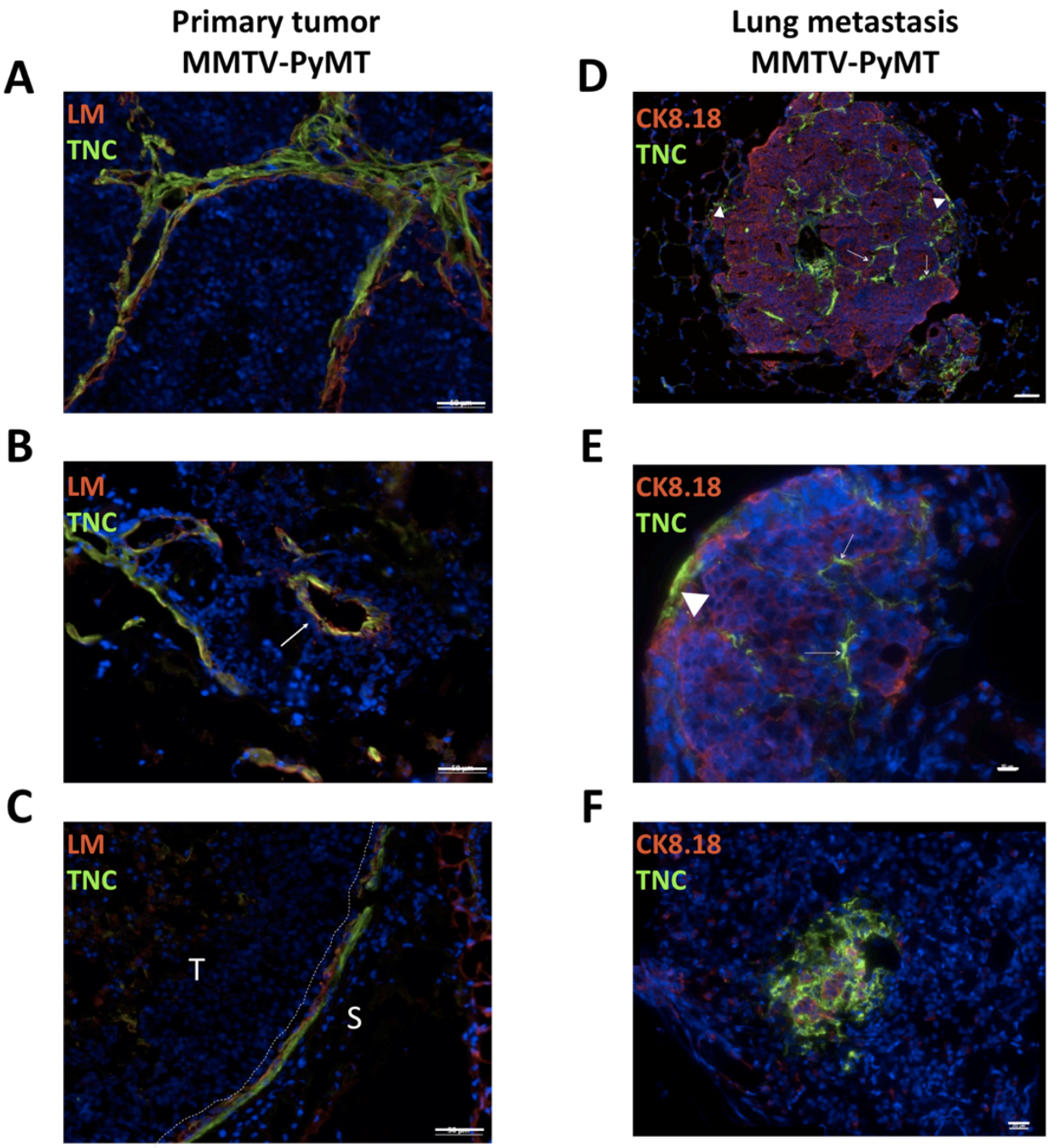


Figure 11. Tenascin C expression in breast tumors and lung metastasis of MMTV-PyMT mice

IF expression analysis of tumor tissue from PyMT mice. Representative pictures are shown.

(A-C) Note that TNC (green) and LM (red) are expressed in PyMT breast tumors ECM tracks **(A)**. **(B)** TNC and LM are expressed around potential blood vessels (arrow). **(C)** TNC and LM are present in the periductal area of PyMT breast tumors. Dotted line marks the limit between the tumor (T) and surrounding stroma (S). Scale bars, 50 µm.

(D-F) TNC expression (green) in PyMT metastatic lung tissue. Cancer cells were identified by their expression of the luminal epithelial cell marker CK8/18 (red). TNC forms tracks mainly located inside the metastasis (arrows in D-E) but sometimes also at the border of the metastasis (arrowheads in D-E). Some PyMT metastatic lungs contain areas with high TNC expression surrounding few CK8/18+ micrometastatic cells are present. Scale bars: D, 50 µm; E, 10 µm, F, 20 µm.

1.2 No impact of TNC on breast tumor progression in compound MMTV-PyMT/mTNC mice

As mentione above several lines of evidence suggest that high TNC expression favors breast tumor malignancy and particularly lung metastasis formation. Nevertheless a causal link between TNC expression and breast tumor aggressiveness has not been demonstrated in a genetic breast cancer model with an intact immune system. This is important since TNC has an important impact on inflammation and the immune system (140) (141). Based on the literature I hypothesized that overexpression of TNC in breast tumors would increase tumor aggressiveness, by promoting breast primary tumor initiation, growth, dissemination and/or metastatic colonization. To test this hypothesis, Martial Kammerer, a former post-doctoral fellow in our laboratory generated a mouse model using the MMTV promoter to ectopically express TNC in the mammary gland.

Expression of the transgene (RNA) in mammary glands from MMTV-mTNC virgin female mice had been verified previously by RT-qPCR (see the Material and Methods section). To determine the effect of ectopic TNC on PyMT breast cancer progression, compound PyMT/mTNC heterozygous mice derived from three founder lines (founders 7 [F7], 9 [F9] and 10 [F10]) had been generated and, tumorigenesis was compared to that of PyMT control mice with wildtype TNC expression. Tumor and metastasis analysis in these mice revealed that ectopic TNC did not modify breast tumor incidence or tumor burden (**Figure 12A**). Histological analysis did not reveal any difference either: all the animals developed breast ductal carcinomas with differentiated cancer cells organized in a typical nodular and cribriform fashion (**Figure 12B**). In addition, ectopic TNC expression did not impact significantly on lung metastasis formation (**Figure 12C**). Altogether these data indicated that ectopic TNC expression does not increase breast tumor progression in PyMT mice. A potential explanation for this unexpected result could be a low expression of ectopic TNC, which has not been addressed in this model but in the MMTV-NeuNT/mTNC model where the same MMTV-TNC mice had been used, and total TNC levels were not increased (see below, 2.2.2).

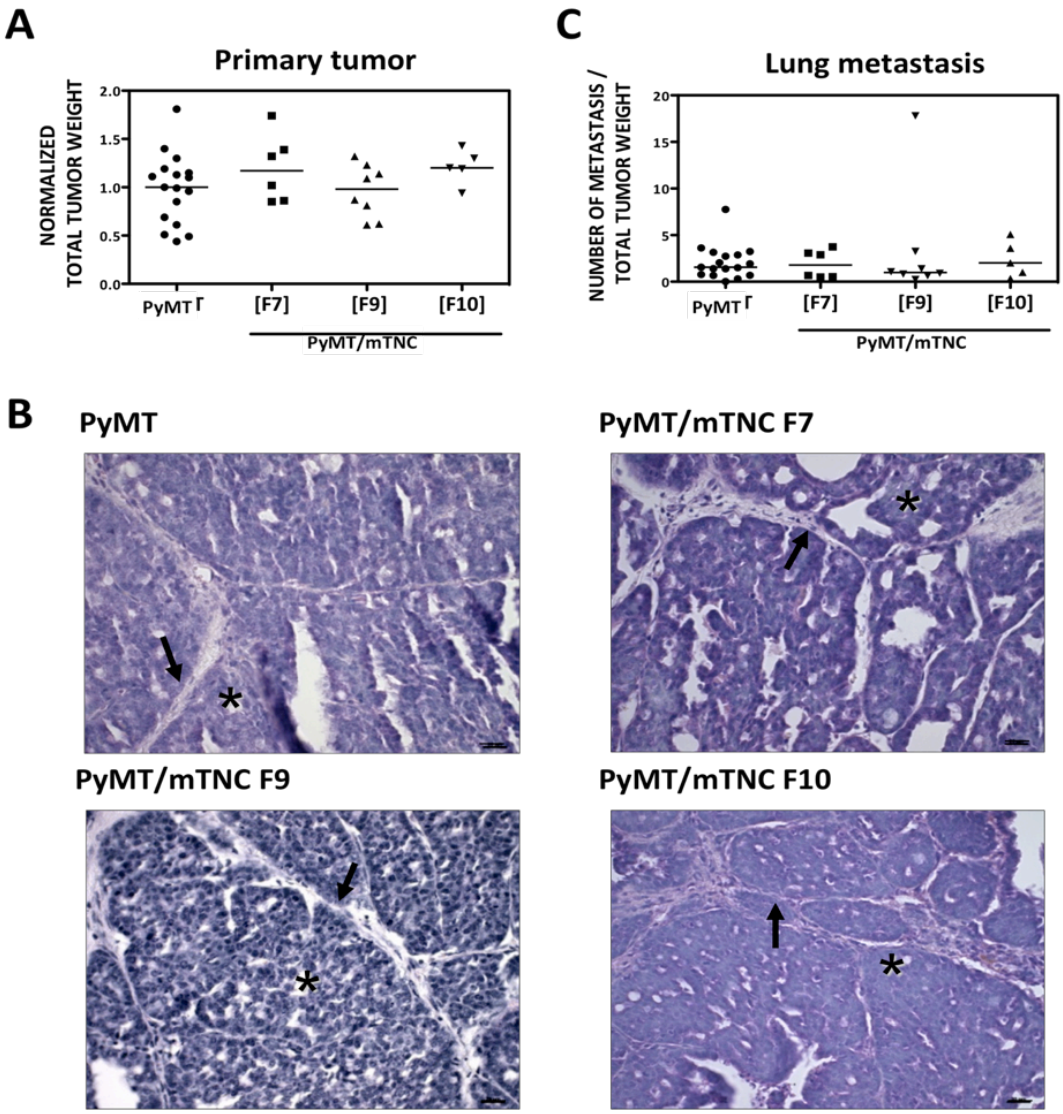


Figure 12. Impact of MMTV-mTNC on tumor progression in compound MMTV-PyMT/mTNC mice

(A) Total tumor weight was determined after 16-17 weeks in control PyMT and three compound PyMT/mTNC lines (founders 7, 9 and 10). Tumor burden was determined for PyMT/mTNC from different founders ([F7] n = 6, [F9] n = 8, [F10] n = 5) and normalized to the mean of the control group (n = 17 PyMT mice). For each group, the bar represents the median of individual values. No significant differences were observed (all p values > 0.05, Mann-Whitney test).

(B) Histological analysis of PyMT and PyMT/mTNC breast tumors. Independent of the founder line, all compound PyMT tumor mice (including the controls) developed invasive breast carcinomas with infiltrated stroma (arrows) and "holes" (marked with asterisks) between the cancer cells that are characteristic of a cribriform histological pattern. Scale bars, 20 μm.

(C) Lung metastasis quantification was determined by histological analysis using a stereological method. The number of metastases quantified was normalized to the total tumor weight per mouse (PyMT n=17, PyMT/mTNC, [F7] n=6, [F9] n=8, [F10] n=5). Bars represent the median of the groups. No significant differences were observed (all p values > 0.05, Mann-Whitney test).

1.3 The absence of TNC does not affect PyMT tumor growth or metastasis

A study of the effect of the absence of TNC in PyMT breast tumor progression was previously carried out by Talts and collaborators in a mixed genetic background (131). These authors had shown that PyMT breast tumor progression is not affected in the absence of TNC, although the organization of the tumors was altered and, more monocytes and macrophages were found in TNC -/- tumors (131). Since the genetic background can have a strong impact on the outcome of an experiment, the experiment was repeated here using a pure FVB genetic background. We had analyzed PyMT mice with 2 alleles of TNC (TNC +/+), only one allele (TNC +/-) or no TNC (TNC -/-) after 10 generations of backcrossing TNC +/- mice with FVB mice (see the Material and Methods section), which could be considered as a pure background. The absence of TNC in PyMT TNC -/- breast tumors was verified by western blot (see the Material and Methods section) and the derived breast tumors and lung metastases were analyzed. This study showed growth (**Figure 13A)** or on the number of lung metastases (**Figure 13C**). Histological analysis of the primary tumors did not display any major difference between tumors of the different genotypes (**Figure 13B**). My results obtained in a pure FVB genetic background thus confirm previous data that were gathered on a mixed genetic background (131), showing that TNC does not affect tumor progression in the PyMT breast cancer mouse model.

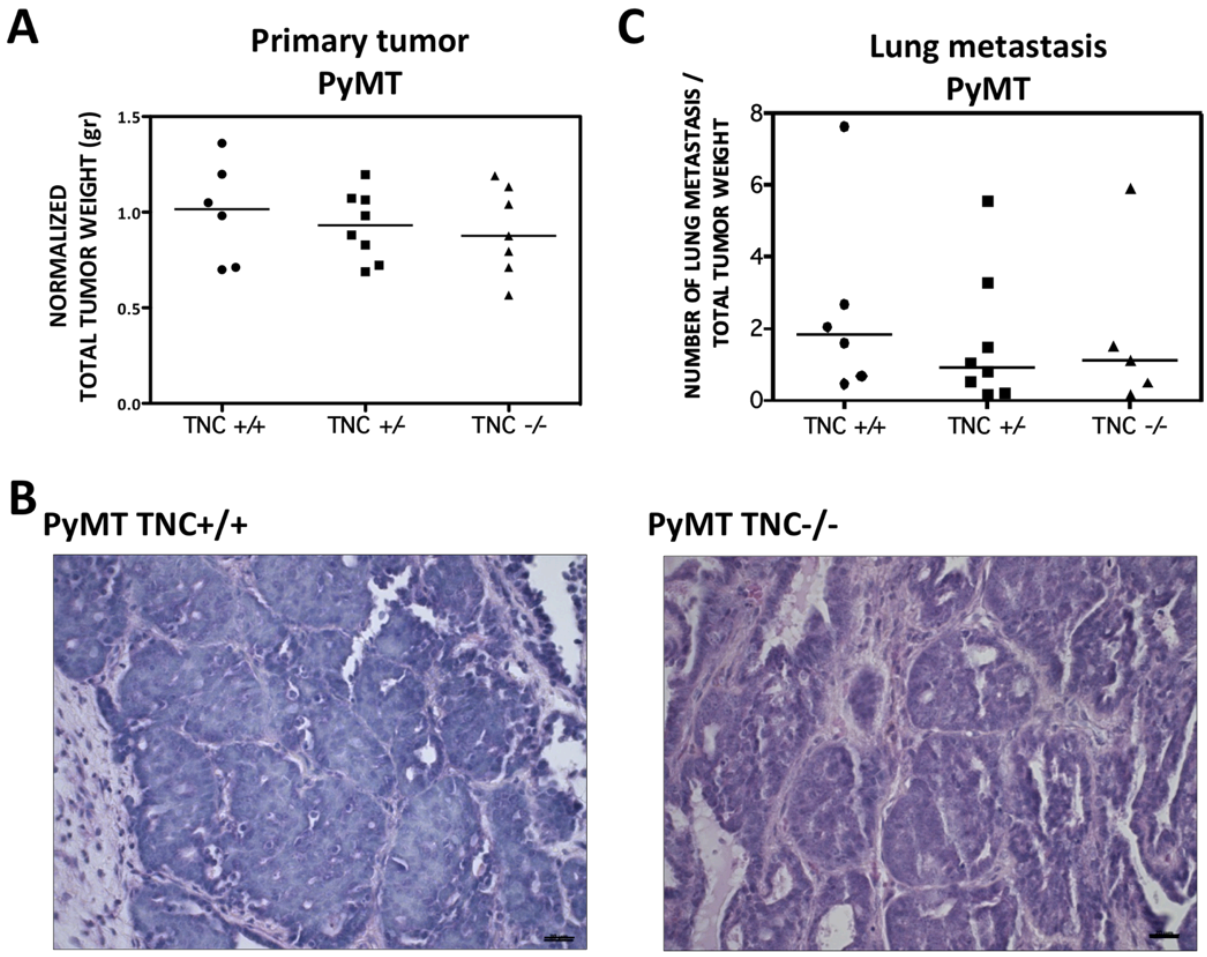


Figure 13. Effect of the absence of TNC on PyMT breast tumor progression

(A) Total tumor weight is shown upon normalization to the mean of the control group (PyMT/TNC +/+). Mice were sacrificed between 110-121 days, TNC +/+ (n=6), TNC +/- (n=8), TNC -/- (n=7). For each group, the bar represents the median of individual values. No significant differences were observed (all p values > 0.05, Mann-Whitney test).

(B) Histological analysis of PyMT TNC +/+ and PyMT TNC -/- breast tumors upon HE staining. Note that both cohorts developed invasive cribriform breast carcinomas. Scale bars, 20 µm.

(C) Histological quantification of the number of lung metastases using a stereological method (PyMT/TNC +/+, n = 6; PyMT/TNC +/-, n = 8, PyMT/TNC -/-, n = 5). Data were normalized to the total tumor weight per mouse. For each group, the bar represents the median of individual values. No significant differences were observed (all p values > 0.05, Mann-Whitney test).

2. IMPACT OF DIFFERENT TNC EXPRESSION LEVELS ON MMTV-NeuNT BREAST TUMOR PROGRESSION

2.1 Description of TNC expression in tumors and lung metastasis of MMTV-NeuNT mice

2.1.1 Specificities of pulmonary metastases observed in NeuNT mice

In line with previous descriptions of the lung metastases occurring in the several MMTV-ErbB2 based murine breast cancer models (65) (76) (142) (143), I confirmed that in our NeuNT mice, a significant proportion of pulmonary metastases was localized within the vasculature as tumor emboli (**Figure 14A**) whereas only few metastases were present in the parenchyma (**Figure 14B**). Interestingly, I observed in a few instances, some tumor cell emboli within the peri-tumoral area in the mammary gland (**Figure 14C**), as previously reported (144), suggesting that emboli could represent a form of tumor cell dissemination in the NeuNT mouse model. As noted in the introduction section, such tumor cell emboli localized in the vasculature are not artifacts of murine tumor models (62) (145) but are also recurrently observed in human breast (and other) cancer patients with clear correlations to poor prognosis for the patient (146) (147) (148) (149).

To gain novel insights into the composition and role of the NeuNT pulmonary intravascular metastases, I immunophenotypically characterized them in more details. First I verified that ErbB2 expressing tumor cells are indeed present in the emboli (**Figure 14D**). I further showed that cells are proliferating as their nuclei stained for Ki67 (**Figure 14E**). These results support the notion that NeuNT tumor emboli are productive and growing metastases. In addition to exclusively intravascular or parenchymal lung metastases, I observed in a few instances a “mixed” phenotype where metastases contained both intravascular and parenchymal neighboring areas: tumor cells were almost “caught in the act” of extravasating (**Figure 14F**). This shows that tumor cells can extravasate from emboli to enter the lung parenchyma, as had already been demonstrated by functional studies (143). Moreover, I quantified the proportion of intravascular *versus* parenchymal metastases at different time points after first primary tumor detection (**Figure 14G-I**). This analysis revealed that shortly (1-4 weeks) after first tumor detection, lung metastases were exclusively intravascular while later the proportion of parenchymal metastases increased (and conversely the proportion of intravascular metastases decreased). This indicates that parenchymal metastases result from the evolution of emboli as previously shown (143).

Altogether, these data confirm that in the NeuNT model, tumor emboli represent an early and transitory stage of lung metastasis, which can then further grow, and/or progress upon extravasation and growth to form parenchymal metastases.

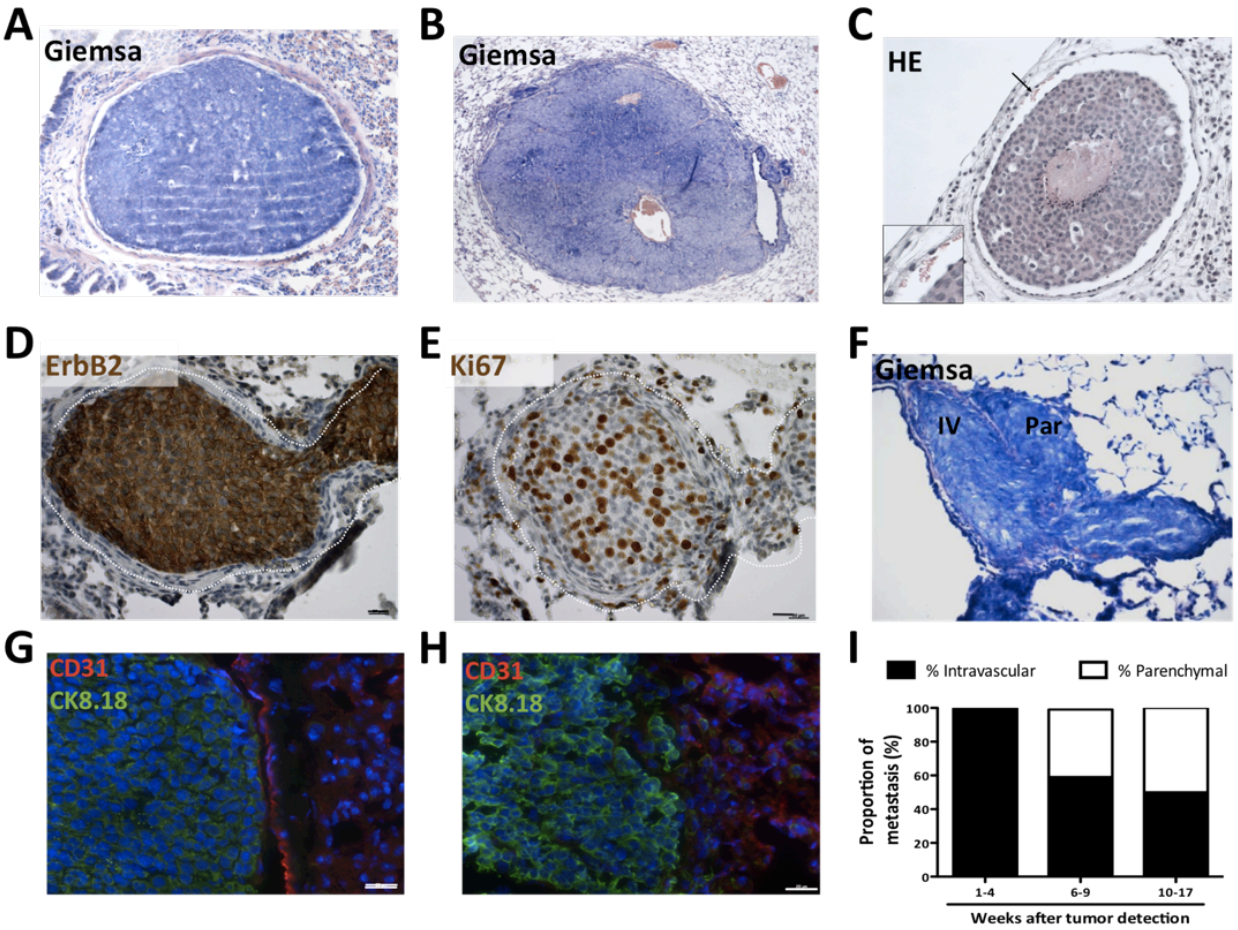


Figure 14. Characterization of lung metastases in NeuNT mice

(A-B) Representative pictures of lung metastasis phenotypes upon Giemsa staining are shown: tumor embolus/intravascular metastasis (A, obj. magnification 20X) and parenchymal metastasis (B; obj. magnification 5X).

(C) A tumor embolus observed in blood vessels near the primary tumor (arrow denote erythrocytes within the blood vessel). An enlargement of this area is shown in the inset (left bottom).

(D-E) Representative pictures of tissue stainings for the indicated molecules. Note that cells in the tumor emboli express ErbB2 (D) and the proliferation marker Ki67 **(E)** demonstrating the tumor nature and the proliferation of the cells within intravascular metastases. Scale bars, 20 µm. **(F)** Example of a "mixed" metastasis phenotype which is characterized by intravascular (iv) and parenchymal areas (Par). Obj. magnification 20X**.**

(G-H) Identification of intravascular **(G)** and parenchymal **(H)** metastases by immunofluorescent analysis of CK8/18 (green) as cancer cell marker and CD31 (red) as a marker of EC/blood vessels.

(I) Percentage values of intravascular and parenchymal lung metastases in NeuNT mice sacrificed at distinct time points after first tumor detection. Analysis of lung metastasis was done in cryosections (1-4 weeks, N = 3 mice, n = 10 metastases; 6-9 weeks, N = 5, n = 18; 10-17 weeks, N = 3, n = 10). Scale bars 20 µm.

2.1.2 Description of TNC expression in NeuNT breast tumors and lung metastasis

To address whether TNC is expressed in NeuNT tumor mice I had analyzed TNC expression in primary tumors and metastatics lungs by IF and IHC in mice of different age. I observed that TNC is expressed in NeuNT breast tumors (**Figure 15**) and their derived lung metastases (**Figure 16**). In primary NeuNT tumors the TNC expression pattern is similar to that in PyMT tumors: inside tumors, TNC is expressed within stromal tracks (**Figure 15A**) and around potential blood vessels (**Figure 15B**), and is also found in the stroma direclty surrounding the tumor (**Figure 15C**).

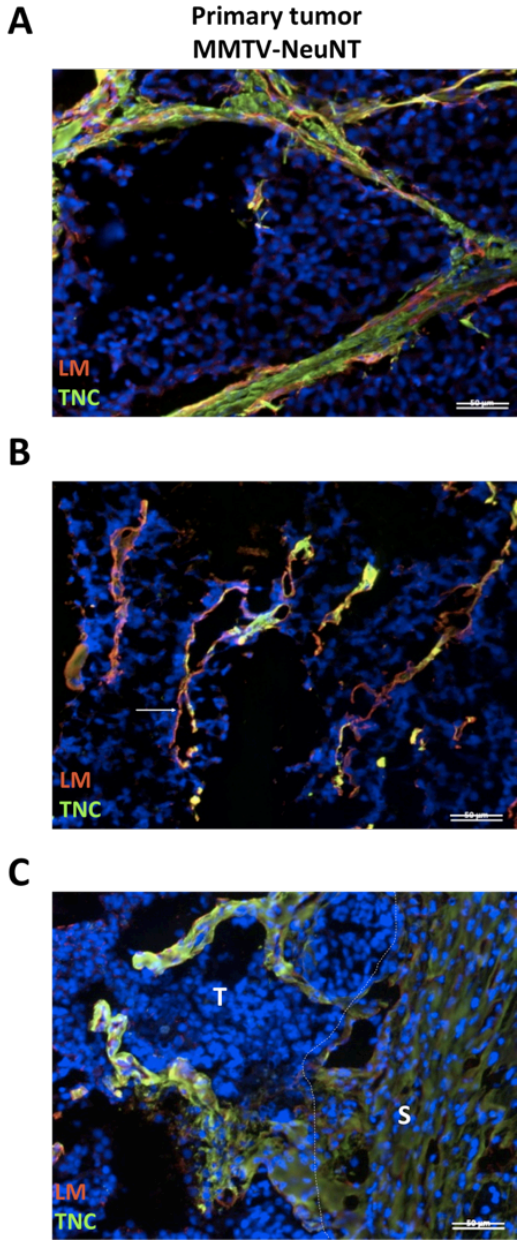


Figure 15. TNC expression patterns in MMTV-NeuNT breast primary tumors

Representative pictures of immunofluorescent analysis for TNC (green) and LM (red). Note that TNC and laminins are expressed in NeuNT breast tumors.

(A) TNC and laminins form tracks inside the tumors.

(B) TNC and laminins are also present around potenial blood vessels. TNC and laminins colocalized in some areas but did not in others. Arrow points a potential blood vessel.

(C) Both proteins are also present in the peritumoral stromal area. The dotted line separates the surrounding stromal area (S) from the tumor (T) area. Scale bars, 50 µm.

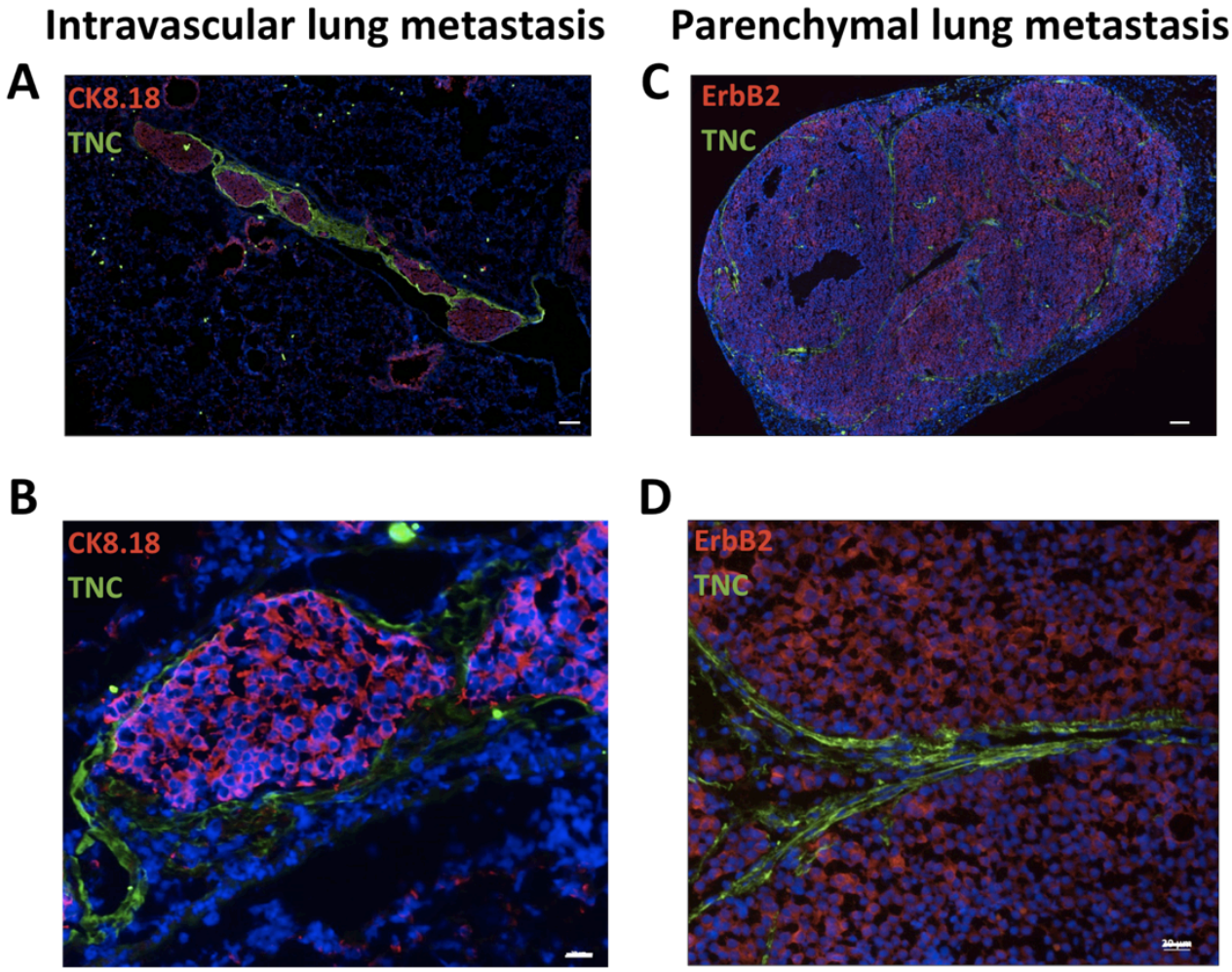


Figure 16. TNC is expressed in intravascular and parenchymal NeuNT lung metastases

Representative pictures of immunofluorescent stainings for TNC (green) and cancer cells (red, CK8/18 or ErbB2) in intravascular **(A, B)** and parenchymal **(B,D)** lung metastases.

(A, B) Note that TNC (green) is expressed at the periphery of tumor cell emboli (red).

(C, D) Note TNC expression in tracks in parenchymal metastasis. Scale bars : 100 µm (A and C), 20 µm (C and D).

Next, I wanted to know whether TNC is expressed in NeuNT pulmonary metastases, which was addressed by tissue staining. Indeed TNC is expressed both in NeuNT tumor emboli and parenchymal metastases (**Figure 16**). In intravascular metastases, TNC is localized around the emboli (**Figures 16A-B, 17A-C**), which in 3 dimensions may be viewed as tumor spheres. Importantly, TNC was found to be expressed in all but one of tumor emboli analyzed in a series of 11 analyzed mice and 24 emboli, suggesting that the majority of tumor emboli express TNC. To investigate whether other ECM molecules are co-expressed with TNC in these intravascular metastases, I analyzed expression of total LM (most isoforms) and FN by IF. Indeed,

LMs and FN are also expressed at the periphery of tumor emboli, in close vicinity to TNC (**Figure 17B-C**). I also observed that ECM tracks composed of TNC and LM or TNC and FN can reach the center of the emboli (**Figure 17B**). The particular ECM expression pattern at the periphery of emboli suggests that tumor emboli are surrounded by an ECM capsule. It is intriguing to speculate that this capsule potentially protects the tumor cells while traveling in the circulation and/or is instrumental in docking onto the lung endothelium before extravasation into the lung parenchyma.

From my first analyses of tumor cell emboli and their content (**Figures 14A, 14D, 16A, 16B**) it was obvious that stromal cells were also part of the NeuNT emboli. To better characterize stromal candidates present in the emboli I performed an IF analysis using established markers for mesenchymal stromal cells (S100A4), EC (CD31), and platelets (CD41). I observed that in addition to cancer cells, S100A4-expressing mesenchymal stromal cells (**Figure 17D, 17G**) are present, in particular at the periphery of the tumor emboli. In addition platelets are present at the periphery of a majority of emboli (**Figure 17E, 17H**). I also often observed a layer of CD31-expressing EC at the periphery of emboli, within the vasculature (**Figure 17F, 17I**), showing that NeuNT tumor cell emboli can be “endothelialized”.

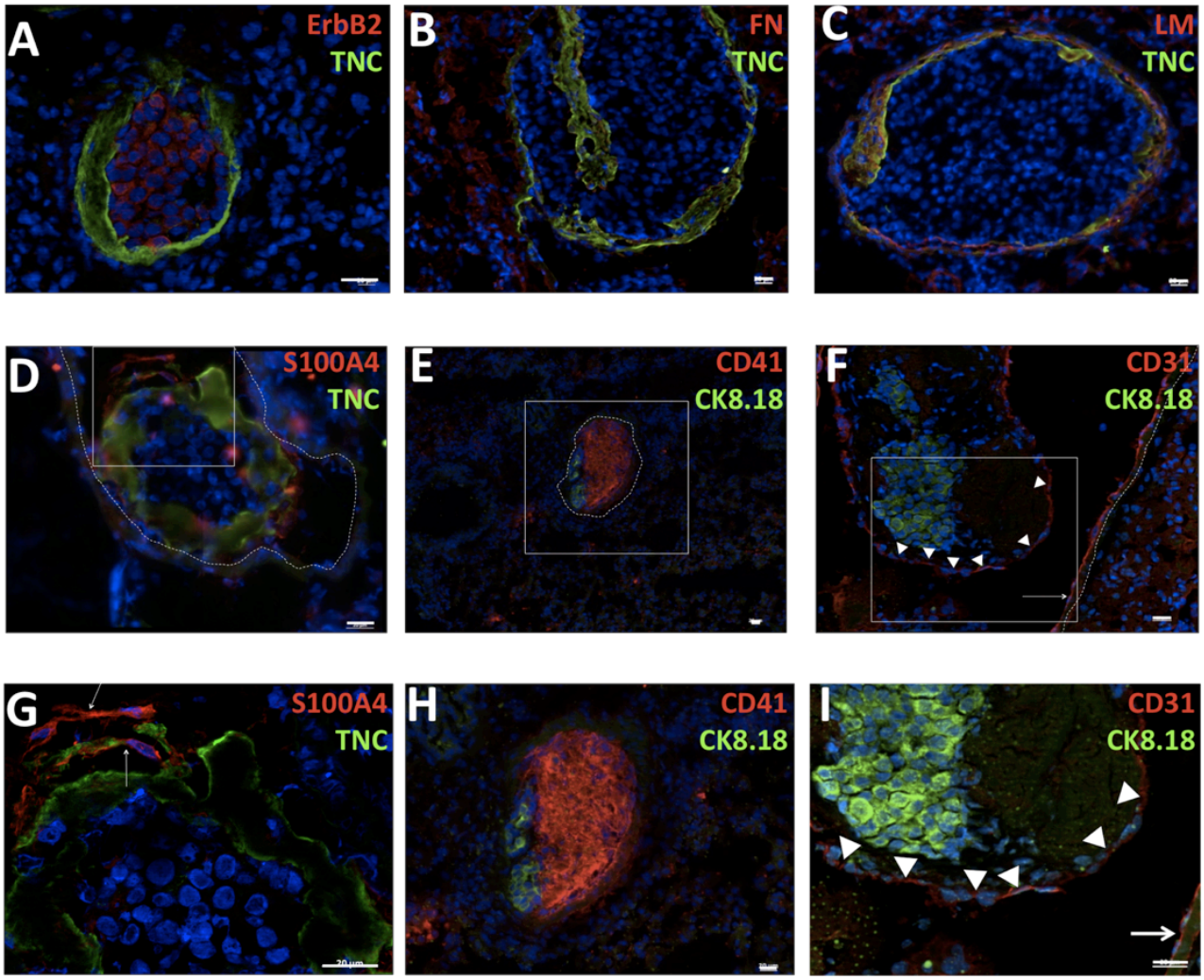


Figure 17. Characterization of lung metastases in NeuNT mice

Representative pictures of immunostainings for the indicated ECM molecules and cellular markers in lung tumor emboli. Note that tumor emboli are largely composed of cancer (ErbB2 positive) cells surrounded by an ECM.

(A-C). Note that this ECM layer contains TNC **(A)** which colocalizes with Fibronectin **(B)** and LM **(C).**

(D-I). Note the presence of S100A4-positive mesenchymal stromal cells **(D, G),** CD41-positive platelets **(E, H)** and CD31-positive endothelial cells **(F, I)** in the tumor emboli. Dotted lines **(D, F**) mark blood vessels. White squares in panels **(D-F)** delineate the fields shown at higher magnification in panels **(G-I**), respectively. **(G)** Arrows points S100A4-positive cells. **(F, I)** The arrowheads points the layer of EC that surrounds the tumor embolus whereas the arrow points the lung blood vessel. Scale bars, 20 µm.

2.2. Impact of TNC in compound MMTV-NeuNT/mTNC mice on breast tumor progression

2.2.1 No impact of TNC on breast tumor progression in compound MMTV-NeuNT/mTNC mice

I had analyzed the effect of ectopic expression of TNC in NeuNT breast tumor progression. Because of the long NeuNT tumor latency (5.4 months on average) I did this analysis only with mice from the MMTV-mTNC founder 9 ([F9]), which is the founder that expressed the highest levels of the transgene mRNA (50-fold above control) in the mammary gland of virgin female mice (see the Material and Methods section). I could not find any difference in tumor incidence (100% of the females developed tumors), tumor latency (**Figure 18A**) or tumor burden (**Figure18**) comparing NeuNT mice with ectopic TNC and wildtype TNC. Histological analysis of the breast tumors revealed no obvious difference in tumor type or cancer cell differentiation (independently confirmed by an expert anatomo-pathologist; **Figure 18C**). To study lung metastasis formation quantitatively, I used two complementary approaches at histological (**Figure 18D**) and molecular (upon quantification of ErbB2 mRNA by RT-qPCR; **Figure 18E**) level. No difference between genotypes was observed.

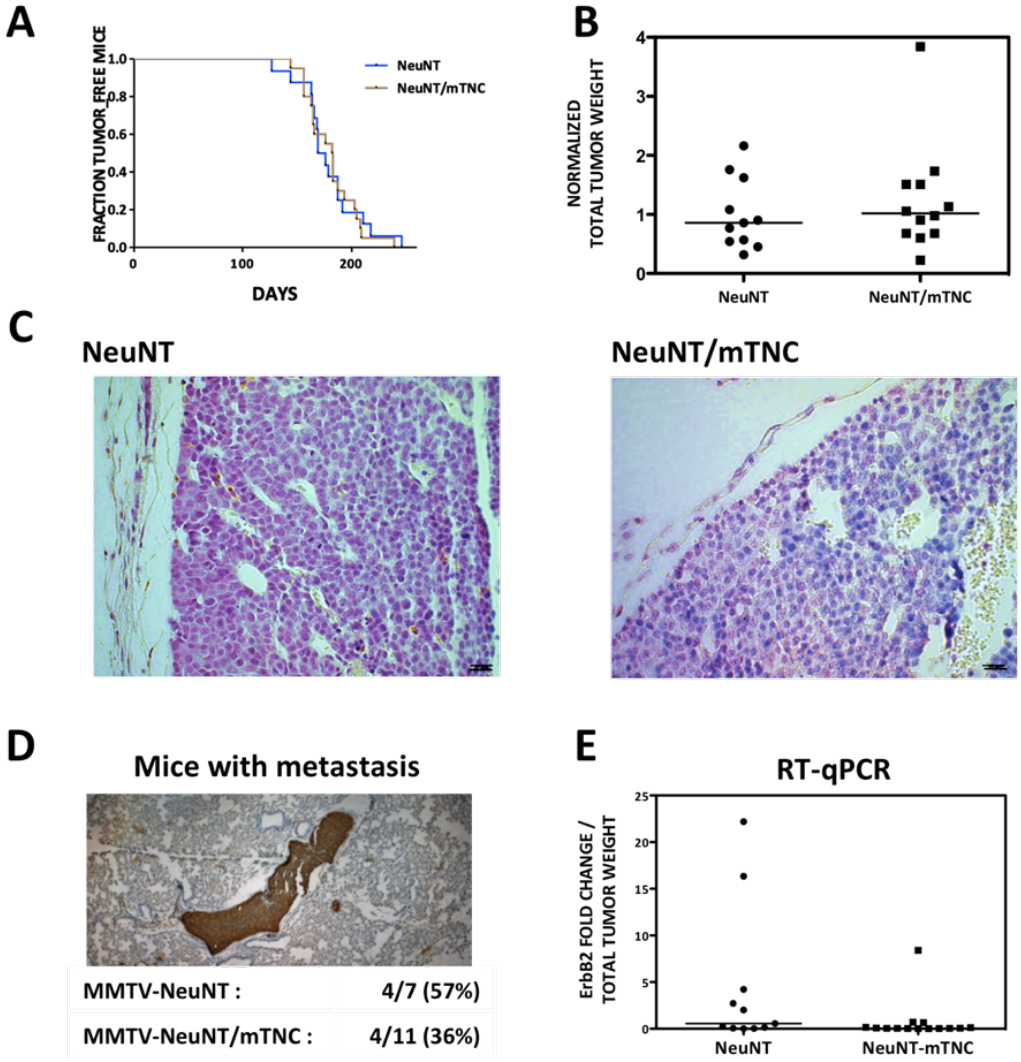


Figure 18. Impact of MMTV-mTNC on NeuNT breast tumor progression

Analysis of primary tumor onset (A), weight (B) and organisation (C) and of lung metastases (D-E) in NeuNT and NeuNT/mTNC mice derived from MMTV-TNC founder line 9.

(A) First tumor appearance in NeuNT (n = 16 mice) and NeuNT/mTNC (n = 20) mice was determined upon twice weekly palpation. No difference was observed between genotypes. (p = 0.774, log-rank test).

(B) Total tumor weight of NeuNT (n = 11) and NeuNT/mTNC (n = 12) mice sacrificed between 4 to 6 weeks after tumor detection. Values were normalized to the mean of the control group ((p = 0.51, Mann Whitney test).

(C) Histological analysis of NeuNT and NeuNT/mTNC breast tumors. NeuNT and NeuNT/mTNC mice developed invasive cribriform breast carcinomas with differentiated cells. Scale bars, 20µm.

(D) Histological quantification of lung metastases from NeuNT (n = 7) and NeuNT/mTNC (n = 11) mice upon ErbB2 staining.

(E) Quantification of the expression of ErbB2 mRNA in lungs by RT-qPCR (NeuNT n = 11; NeuNT/mTNC n= 14). Data were normalized to tumor burden. For each group the bar represents the median of individual values. No significant difference was observed (p = 0.067, Mann Whitney test).

2.2.2 Analysis of total TNC levels in compound MMTV-NeuNT/mTNC tumors

A previous analysis in our laboratory by M. Kammerer had shown that the transgene MMTV-mTNC-IRES-Luc is expressed in the mammary gland of virgin MMTV-mTNC mice. But it has not been addressed if the transgene is still expressed in breast tumors, and most importantly if the expression of the transgene is exceeding the endogenous TNC levels which was assumed by analogy to many other transgenic models. Therefore, I analyzed the expression of the transgene by RT-qPCR in breast and lung tissue of NeuNT and NeuNT/mTNC mice. By using primers specific for the transgene I did not detect a signal in NeuNT breast tumors, whereas the C_t values in NeuNT/mTNC tumors and lung tissue were around 30,, indicating detectable expression of the transgenic mRNA. By contrast no change in expression of the housekeeping gene RLP19 was seen between samples. Thus transgenic TNC mRNA is expressed in both the primary tumors and lung metastasis of NeuNT/mTNC mice. With primers specific for endogenous TNC I addressed whether expression of the transgene had an impact on the expression of endogenous TNC. But the mRNA levels of endogenous TNC were the same in both genotypes (breast tumors, **Figure 19A**; metastatic lungs, **Figure 19C**) suggesting that the transgene had no impact on the RNA expression levels of endogenous TNC. By using primers that detect both endogenous and transgenic TNC I observed that mRNA levels of transgenic TNC do not significantly increase the total mRNA levels of TNC (**Figure 19B, 19D**). To determine TNC protein levels, a western blot analysis was done. This analysis showed that in the analyzed 6 double transgenic NeuNT/mTNC tumors rather less total TNC protein was expressed than in the 6 control tumors (**Figure 19E-F**). Whether this difference is statistically significant would need to be addressed in more samples, but this observation potentially points at a negative impact of the transgene on total TNC protein levels. In summary these data indicate that although the TNC-encoding transgene is expressed at RNA level in NeuNT breast tumors and lung metastasis, transgenic TNC did not exceed the endogenous levels and may even have a negative impact on total TNC protein levels. This could explain why no effect on tumor onset, growth and lung metastasis was seen in compound MMTV-NeuNT/mTNC mice in comparison to NeuNT mice.

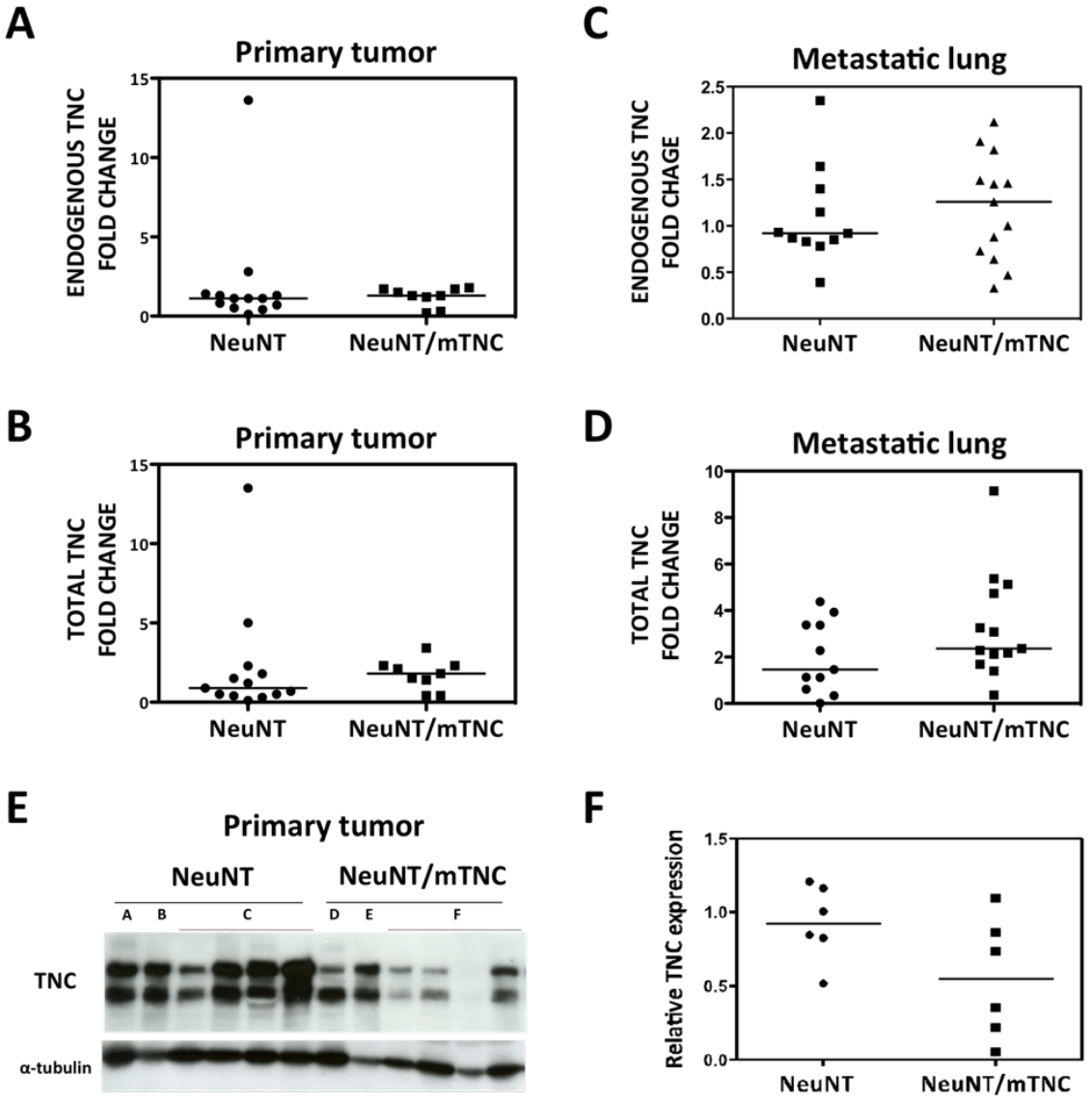


Figure 19. TNC expression in tumors and metastatic lungs of NeuNT/mTNC mice

Expression of endogenous and transgenic TNC in breast tumors (**A, B, E and F)** and in metastatic lungs **(C, D)** of NeuNT and NeuNT/mTNC mice was done by RT-qPCR **(A-D)** and Western blot **(E).**

(A, B) Breast tumors from NeuNT (N = 8 mice, n = 13 tumors) and NeuNT/mTNC (N = 6 mice, n = 9 tumors) mice sacrificed two weeks after tumor detection. No difference in endogenous TNC levels (A, p = 0.383) or total TNC expression levels (B, p = 0.348) was observed.

(C, D) The expression of TNC was also analyzed in lungs of NeuNT (n = 11 mice) and NeuNT/mTNC mice (n = 13 mice) sacrificed 4-6 weeks after first tumor detection. Note no difference in endogenous TNC expression (C, p = 0.64) or total TNC (**D,** p = 0.1475). The median of each group is shown. Data were analyzed using the Mann Whitney test.

(E, F) Western Blot of breast tumor lysates from NeuNT and NeuNT/mTNC (N = 3 mice, n=6 tumors for both genotypes) mice **(E)** and quantification of signals by densitometry **(F).** In panel E, the letters (A to F) represent different mice, each lane was loaded with a single tumor sample. **(F)** Western blot quantification was done by densitometry of the TNC signal upon normalization to the loading control alpha-tubulin. For each group, the bar represents median relative TNC expression (p = 0.13, Mann- Whitney test).

2.3 Effect of the absence of TNC on NeuNT breast tumor progression

2.3.1 The absence of TNC in NeuNT mice impacts on tumor latency

To address whether the absence of TNC has an impact on tumorigenesis and progression I determined tumor latency, tumor incidence and tumor burden in NeuNT mice with two (TNC +/+), one (TNC +/-) and no (TNC -/-) alleles of TNC. As previously mentioned in the methods section, 129sv TNC -/- mice were initially obtained and therefore I first crossed them (TNC +/-) for 10 generations with FVB mice to obtain TNC -/- on an almost "pure" FVB background. Subsequently, generation 10 FVB NeuNT TNC +/- male mice were bred with TNC +/- females. Therefore, 1 mouse out of 16 was expected to be a female NeuNT TNC -/- mouse. Coupled to the fact that our whole institute experienced breeding issues when these animals were generated, I could only obtain six female NeuNT TNC-/- mice for analysis.

Both NeuNT TNC +/+ and NeuNT TNC -/- mice developed breast tumors with a 100% penetrance but TNC -/- tumor mice developed them with a significantly delayed latency (**Figure 20A**; TNC +/+: t_{50} = 183 days; TNC+/-: t_{50} = 183.5 days; TNC -/-: t_{50} = 232 days). Tumor burden was determined 3 months after palpation of the first tumor, giving rise to a total age of mice ranging from 135 to 198 days in NeuNT TNC +/+ and from 175 to 322 days in NeuNT TNC -/- mice. Analysis of the tumor burden (determined as total tumor weight) did not reveal a significant difference between genotypes (**Figure 20B**).

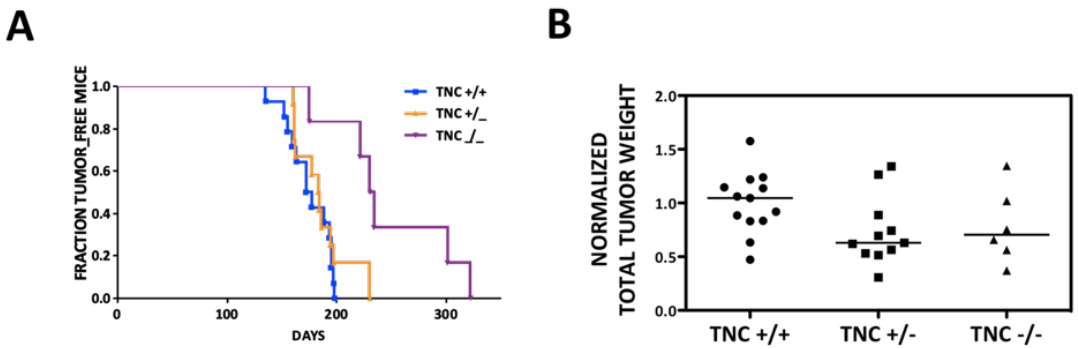


Figure 20. Effect of the absence of TNC on NeuNT breast tumor progression

(A) Fraction of tumor-free mice is shown for NeuNT tumor mice with two (TNC +/+, N=14 mice), one (TNC +/-, N=12) and no (TNC -/-, N=6) TNC wildtype alleles. Determination of first tumor detection was done by palpation of the mammary glands twice per week. The absence of TNC significantly delays tumor latency (TNC +/+ vs TNC +/- p = 0.3886; TNC +/+ vs TNC -/- p = 0.0011; TNC+/- vs TNC -/- p = 0.0115; Log-rank tests). Median tumor-free survival times: TNC +/+, 174.5 days; TNC +/-, 183.5 days; TNC -/-, 232 days.

(B) NeuNT tumor mice (TNC +/+ n = 13, TNC +/- n = 11, TNC -/- n = 6) were sacrificed 3 months after first tumor detection and tumor burden was determined and normalized to the mean tumor burden of the control group (TNC +/+). Note that the tumor burden is not significantly different between NeuNT TNC +/+ and TNC -/- (TNC +/+ vs TNC -/- p= 0.17; TNC +/+ vs TNC +/- p = 0.06; TNC +/+ vs TNC +/- p = 0.61, Mann Whitney test).

Histological analysis of tumor tissue from these mice did not reveal any detectable difference either (**Figure 21A**). NeuNT TNC +/- animals also developed tumors with similar latency and burden as the controls, presumably because TNC expression in TNC +/- breast tumors is comparable to TNC +/+ control tumors (see Material and Methods section). Therefore, subsequently TNC+/- tumor mice were not further analyzed. These results indicate that i) TNC plays an important role(s) during the initial steps of NeuNT tumorigenesis (before tumors are palpable) and ii) that TNC is then dispensable for primary NeuNT breast tumor growth. The latter could be explained either because TNC exerts no specific function during this/these phase(s) of NeuNT breast primary tumor growth or through compensatory mechanisms (see discussion).

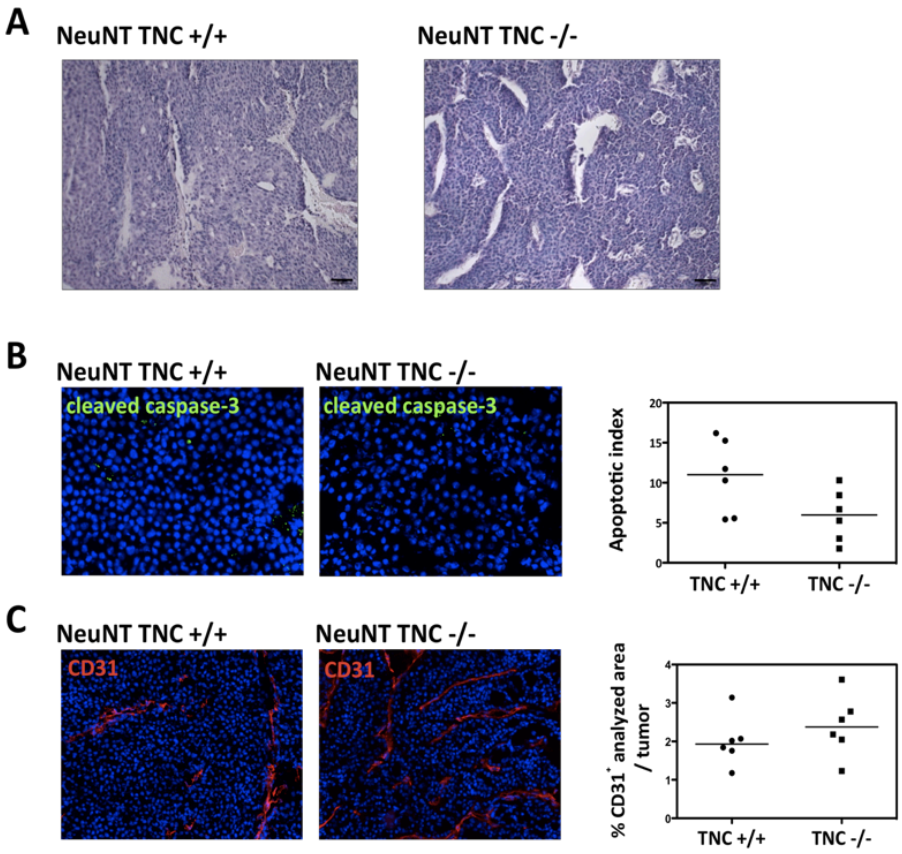


Figure 21. Impact of TNC absence on NeuNT primary breast tumor growth

(A) Histological analysis of NeuNT, TNC +/+ and TNC -/- breast tumors was done upon hematoxylin-eosin staining of tissue sections. Scale bar 50 µm.

(B, C) Representative immunostainings of NeuNT TNC +/+ (N = 6 mice, n = 6 tumors) and TNC -/- (N=6 mice, n = 6 tumors) breast tumors. Muliple frames (8 to 37) were analysed for each sample depending on tissue availability. Right panels: quantification of the staining signals. Individual data points represent the average for each sample/mouse, the bar the median for each group. **(B)** The apoptotic index (number of cleaved caspase-3 positive signals normalized to the analyzed area) is reported. No significant difference was observed (p = 0.093, Mann Whitney test). **(C)** The percentage of CD31+ signal normalized per area was determined. No significant difference was observed (p = 0.24, Mann Whitney test).

Eventhough there was no primary tumor growth defect detectable in NeuNT TNC -/- mice, I analyzed apoptosis and angiogenesis in the primary tumors to cover the possibility that TNC potentially plays a role in NeuNT tumor angiogenesis (see **Figure 15B**) that altogether could explain the effects seen on lung metastasis (see next paragraph 2.3.2, **Figure 23E**). Quantification of IF stainings for cleaved caspase-3 (apoptosis marker) and CD31 (EC marker) revealed no significant difference in either parameter (**Figure 21B-C**).

To get some more insights into the molecular and cellular mechanisms potentially involved downstream of TNC in NeuNT breast cancer progression, we decided to perform a comprehensive (genome-wide) gene expression analysis using microarrays. Therefore I extracted RNA from primary tumors in order to compare the transcriptome of NeuNT primary tumors from TNC+/+ and TNC-/- mice (**Figure 22A**). Although there was no obvious phenotype in primary tumor growth, this choice was made as a rapid way to potentially pinpoint novel molecular candidates. The raw results were obtained only very recently and interestingly, it seems that a limited number of genes is deregulated (**Figure 22B-C**). In-depth analyses are currently ongoing in the lab. This study could provide novel important clues to the roles of TNC (see discussion).

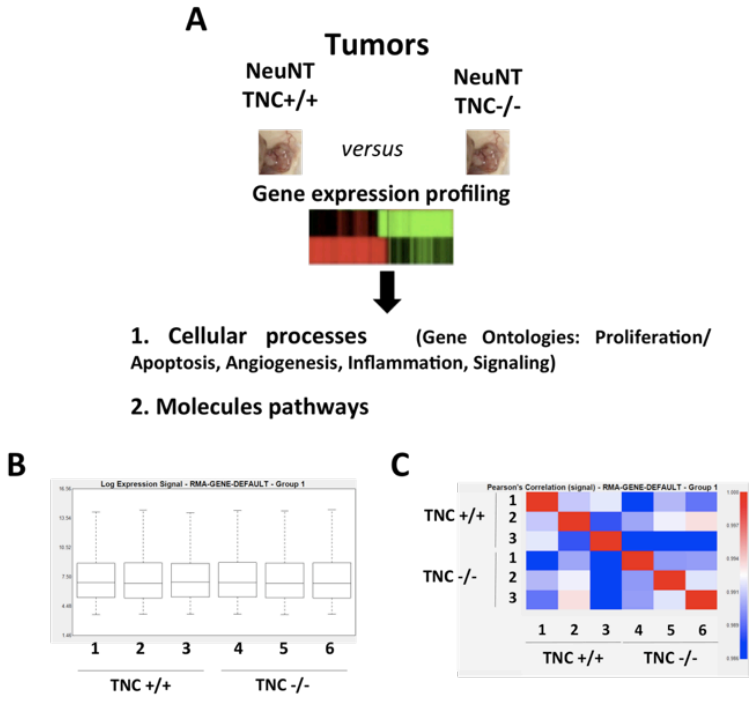


Figure 22. Microarray profiling of NeuNT breast primary tumors

(A) Overview of the approach and aims.

(B) Distribution of signals (log2) after RMA normalization of the raw data obtained from the 3 arrays used to profile TNC+/+ samples (1 – 3) and TNC-/- samples (4-6).

(C) Graphical representation of the inter-individual array correlations (Pearson) of expression signals. Overall, good correlations are observed within each group/genotype. Lower correlations between arrays from different genotypes (TNC +/+ and TNC -/-) are observed, but overall still high. This suggests that the absence of TNC may lead to the deregulation of a limited set of genes.

Anaysis carry out by Thomas Hussenet

2.3.2 Impact of TNC absence on NeuNT lung metastases

I investigated whether the absence of TNC had an impact on lung metastasis formation in MMTV-NeuNT mice by histological analysis of lung tissue using an adapted conventional histological stain (Giemsa). Of note, the left (biggest) lung lobe was systematically analyzed, using a stereological approach. This analysis showed a tendency (not statistically significant) towards less metastasis in TNC-/- tumor mice (**Figure 23A**). Interestingly, among the 6 TNC -/- mice analyzed, 5 had very few metastases while one had the most of the whole series. This latter result suggests that dissemination may not be impaired in TNC-/- animals.

Upon determination of the cumulated metastasis burden (total metastatic area normalized to the total lung tissue area examined) I observed that in the absence of TNC, the overall metastatic burden was reduced (**Figure 23B**). Together with the previous observation, this suggested that eventhough numerous metastatic emboli were present in the lung of one TNC -/- mouse, the growth of TNC-/- emboli was severely impaired.

Quantifying the size of individual emboli indeed revealed that their size (surface) was approximately 2-fold reduced in TNC -/- mice (**Figure 23C**). To understand this difference in size I analyzed the levels of cell proliferation and apoptosis in the lung metastases. I observed that the number of proliferative cells in metastatic emboli was not different between genotypes (**Figure 23D**). However, upon staining for cleaved caspase-3 and quantification, I found that significantly more apoptotic cells (4-fold in average) were observed within TNC -/- emboli (**Figure 23E**). This observation suggests that TNC exerts a protective function for tumor cells to promote their survival in the blood vessels. An increased number of apoptotic cells in the metastatic intravascular lung emboli of NeuNT TNC -/- mice (**Figure 23E**) can explain the reduced embolus size (**Figure 23C**). Finally, in the absence of TNC the percentage of parenchymal metastases was lower than in TNC +/+ animals, 30% of parenchymal metastases in TNC +/+ mice versus 11% in TNC-/- mice (**Figure 23F**). This result suggests that TNC promotes the transition from embolus to extravasated parenchymal metastasis. It will be therefore of further interest to model and determine if this occurs through EMT-promoting signals such as TGF-beta (143) and/or other mechanisms.

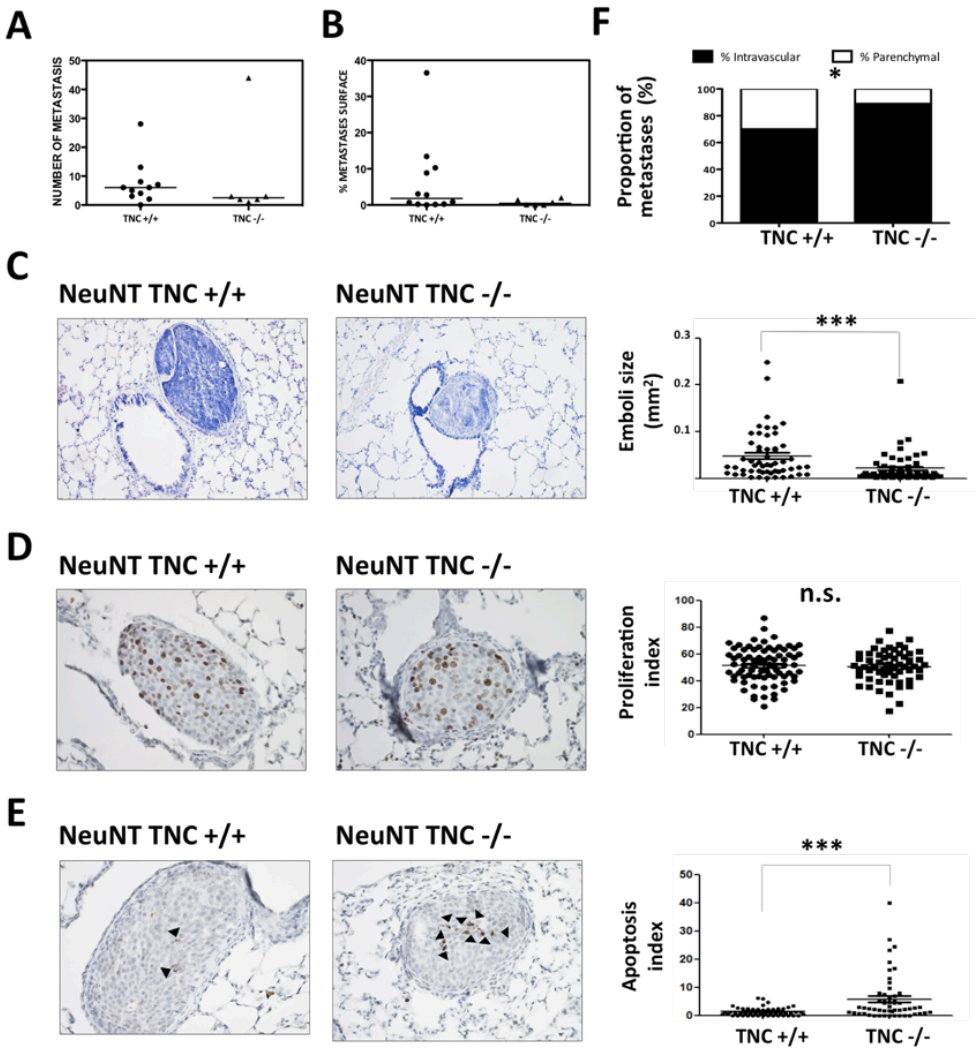


Figure 23. Analysis and quantification of lung metastasis in NeuNT TNC +/+ and NeuNT TNC -/- mice

(A, B) Quantification of the number of lung metastases **(A)** and of the cumulated metastatic burden (metastatic area normalized to total area) **(B)** in lungs of NeuNT TNC +/+ (n = 11) and NeuNT TNC -/- (n = 6) mice that had been sacrificed 3 months after tumor detection.

(C) Giemsa stained lung tissue was used for determination of emboli size (TNC +/+: N= 6 mice and n = 54 emboli; TNC -/-: N=6 mice and n= 54 emboli). NeuNT tumor emboli are significantly smaller in TNC -/- mice (2.2-fold in average; *** $p < 5.10^{-3}$, unpaired Student t test).

(D) The percentage of proliferative cells (Ki67+) per lung metastasis was determined by immunohistochemistry (TNC +/+: N= 6 mice and n = 79 metastases; TNC -/-: N=6 mice and n= 59 metastases). No significant (n.s.) difference was observed (unpaired Student t test). Results were similar when only considering emboli or parenchymal metastases (not shown).

(E) Immunohistochemistry for cleaved caspase-3 was used to determine apoptotic levels in tumor emboli (TNC +/+: N= 6 mice and n = 53 emboli; TNC -/-: N=6 mice and n= 54 emboli). The apoptotic index represents the number of apoptotic cells normalized per embolus area. More apoptotic cells are observed in TNC -/- emboli (4.2-fold in average, *** $p < 10^{-4}$, unpaired Student t test). Arrowheads denote cleaved caspase-3 positive apoptotic cells.

(F) Proportions of intravascular *versus* parenchymal metastases for each genotype (TNC +/+ N= 6 mice, n = 57 emboli and 20 parenchymal metastases; TNC -/-, N = 6 mice, n = 45 emboli and 4 parenchymal metastases.) * $p < 0.02$, Fisher's exact test.

Data obtained and analyzed by Thomas Hussenet.

2.4 EFFECT OF THE ABSENCE OF TNC ON EARLY BREAST TUMOR LESIONS IN NEUNT TUMORS

As noted earlier, all NeuNT TNC -/- mice developed palpable tumors, but with a significant delay (**Figure 24A**). To confirm this phenotype and get a better understanding of the involved mechanisms, we decided to perform an additional study using the NeuNT transgenic model. The goal was to evaluate the role of TNC during the earliest steps of tumorigenesis, before tumors are palpable. To this end I generated a novel cohort of NeuNT TNC+/+ (N=11) and NeuNT TNC-/- (N=13) mice.

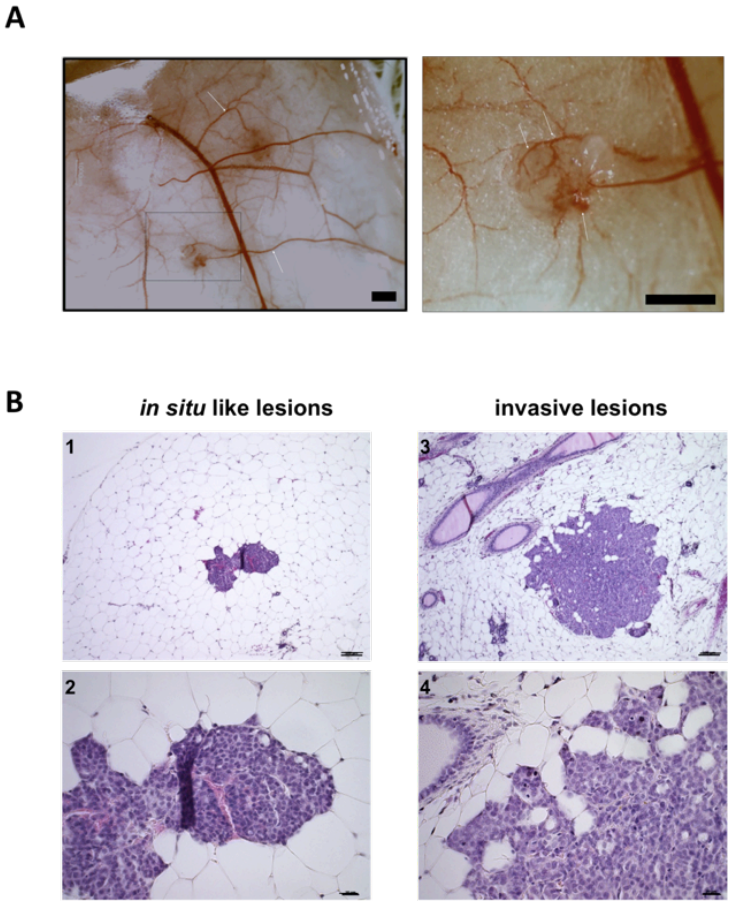


Figure 24. Analysis of early tumor lesions in the mammary gland of NeuNT mice

(A) NeuNT TNC +/+ and NeuNT TNC -/- mice were sacrificed at the age of 4.5 months when no tumors were yet palpable. Note small tumor lesions (enclosed by dotted lines) that are already significantly vascularized (arrows). Note also that one mammary gland can develop multiple tumors (two in this case).

(B) Analysis of early tumor lesion morphology upon HE staining of issue. Representative examples of *in situ*-like lesions (1 and 2) and invasive lesions (3 and 4) are shown. 2 and 4 are higher magnification pictures of 1 and 3 respectively. Scale bars: A, 1 mm; B pictures 1 and 3: 100 µm; B pictures 2 and 4: 20 µm.

In a pilot study, I analyzed three 3-months old NeuNT TNC +/+ mice using whole mount carmine alum stained mammary glands. However, few early small tumor lesion were observed in any of these mice. Therefore we decided to analyze all mice at the age of 4.5 months (before tumors are palpable). During dissection of these mice, I realized that even at this early time point some mice already had small (around 1mm in diameter) but macroscopically visible tumors with evident blood supply (**Figure 24A**). The mammary glands of each mouse were systematically sampled for further analyses (for whole mount, OCT embedding / freezing and fixing/paraffin-embedding).

First histological analysis of some control (NeuNT TNC +/+) mammary glands revealed that both *in situ* and invasive lesions of heterogeneous size are observed at this early time point (**Figure 24B**). To perform qualitative and quantitative analyses of these early tumor lesions, I used

(i) the whole mount preparations, to provide an overview of the mammary gland organization and to quantify the number of lesions,

(ii) the paraffin-embedded and fresh-frozen tissue to perform quantification and immunohistological analyses.

Using whole mount preparations I observed that ductal branching was very heterogeneous. This variability was observed in both TNC +/+ and TNC -/- mice without any clear difference between the genotypes. Since ”nodules” up to 200 μm in diameter were observed in whole mount preparations of mammary glands from normal (without tumors) age-matched and virgin female FVB mice (not shown), I only considered such nodules as early tumor lesions when they were bigger than 200 μm. Such early tumor lesions were observed in whole mount preparations of 63% of TNC +/+ and 50% of TNC -/- mice, and no difference in the number of lesions between genotypes was observed (**Figure 25A**). I also used paraffin-embedded tissue to quantify the number of early tumor lesions (which was normalized to the analyzed area). Also by using this approach I did not observe a significant difference between genotypes (**Figure 25B**). However I realized that the distribution of the number of lesions between genotypes seemed different. About one third of the analyzed NeuNT TNC +/+ mice did not develop lesions, one third presented 1 to 3 lesions and one third developed more than 3 lesions. This distribution was different in TNC -/- female mice of which around half did not develop any lesion, one third developed 1 to 3 lesions and less than 20 per cent developed more than 3 lesions (**Figure 25B, 25D**). These differences were not statistically significant but the same trend was observed for the two independent assessments. Overall, these results suggest a tendency of NeuNT TNC -/- animals to develop lesions later. To further test this possibility, more mice would need to be analyzed.

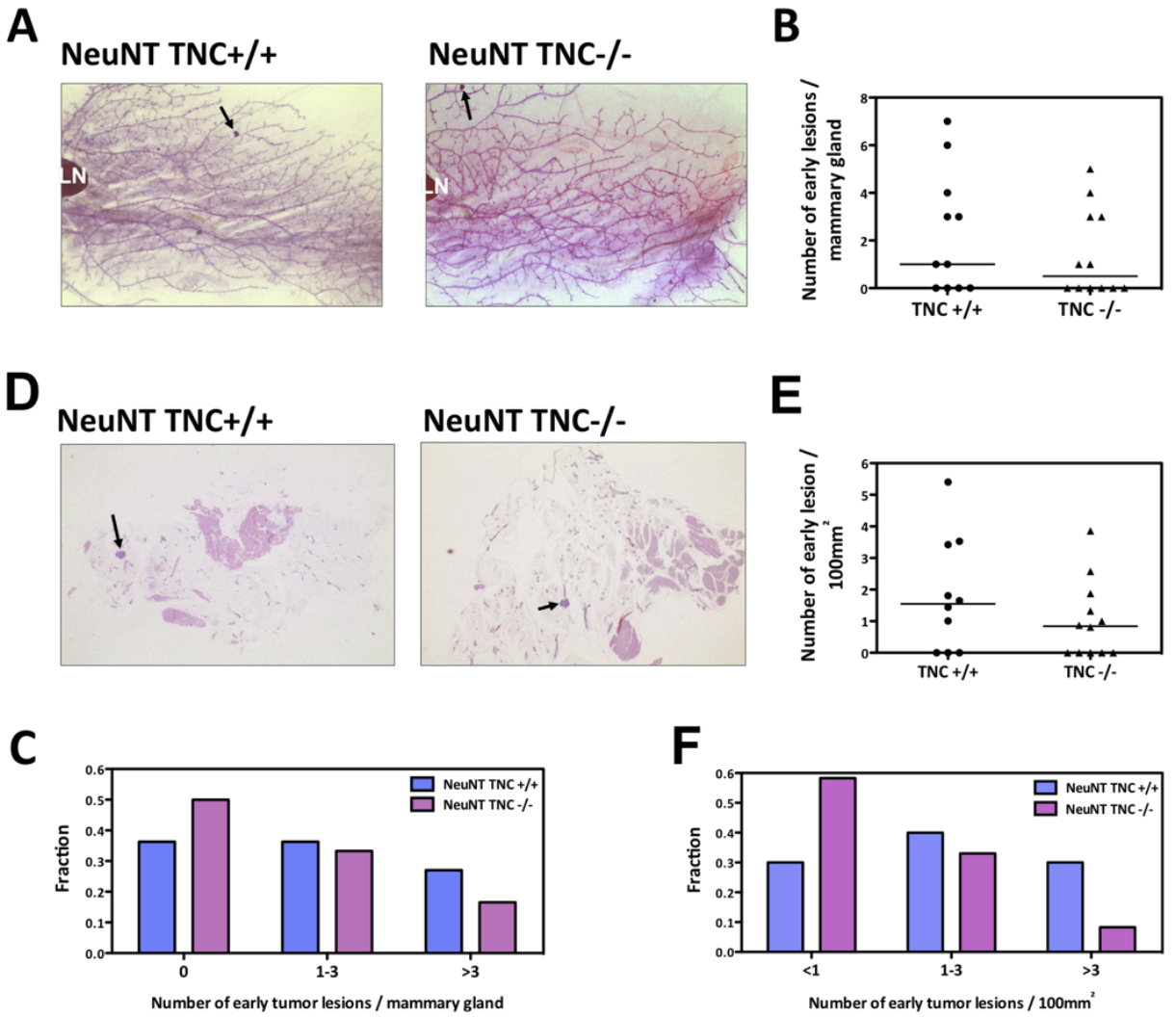


Figure 25. Quantification of the number of early tumor lesions in NeuNT TNC +/+ and NeuNT TNC -/- mammary glands

(A) The 4th right mammary glands of NeuNT TNC +/+ (n = 11) and NeuNT TNC -/- (n = 12) mice were examined upon whole mount preparations. Images show overviews of whole mount preparations. LN indicates the lymph node and arrows point at one early tumor lesion present in each mammary gland.

(B) Quantification of the number of early tumor lesions per mammary gland from the analysis in (A) (p = 0.44, Mann-Whitney test).

(C) The distribution of the data presented in (B), showing the proportion of NeuNT mice (TNC +/+ and TNC -/-) that did no present any tumor lesions, between 1 to 3 or more than 3 lesions (Chi-square test, p = 0.75).

(D) The 2nd and 3rd right mammary glands from the same mouse were recovered, fixed and embedded in paraffin, NeuNT TNC +/+ (n = 10) and NeuNT TNC -/- (n = 12). HE staining was done to visualize small tumor lesions. Arrows point at one early tumor lesion present in each mammary gland. The number of early lesions per 100 mm^2 of tissue is presented in (E) (p = 0.2792, with Mann Whitney test).

(F) The distribution of the data presented in (E), showing the proportion of NeuNT mice (TNC +/+ and TNC -/-) that present >1, between 1 to 3 and more than 3 lesions per 100 mm^2 tissue (Chi-square test, p = 0.29).

Since the number of early tumor lesions is not affected by the genotype I considered that the difference in tumor latency could be a result of a difference in growth of the lesions. To address this possibility I determined the size of the early lesions observed in whole mount preparations (**Figure 26A**) and in paraffin embedded tissue (**Figure 26B**). Nevertheless no difference was observed.

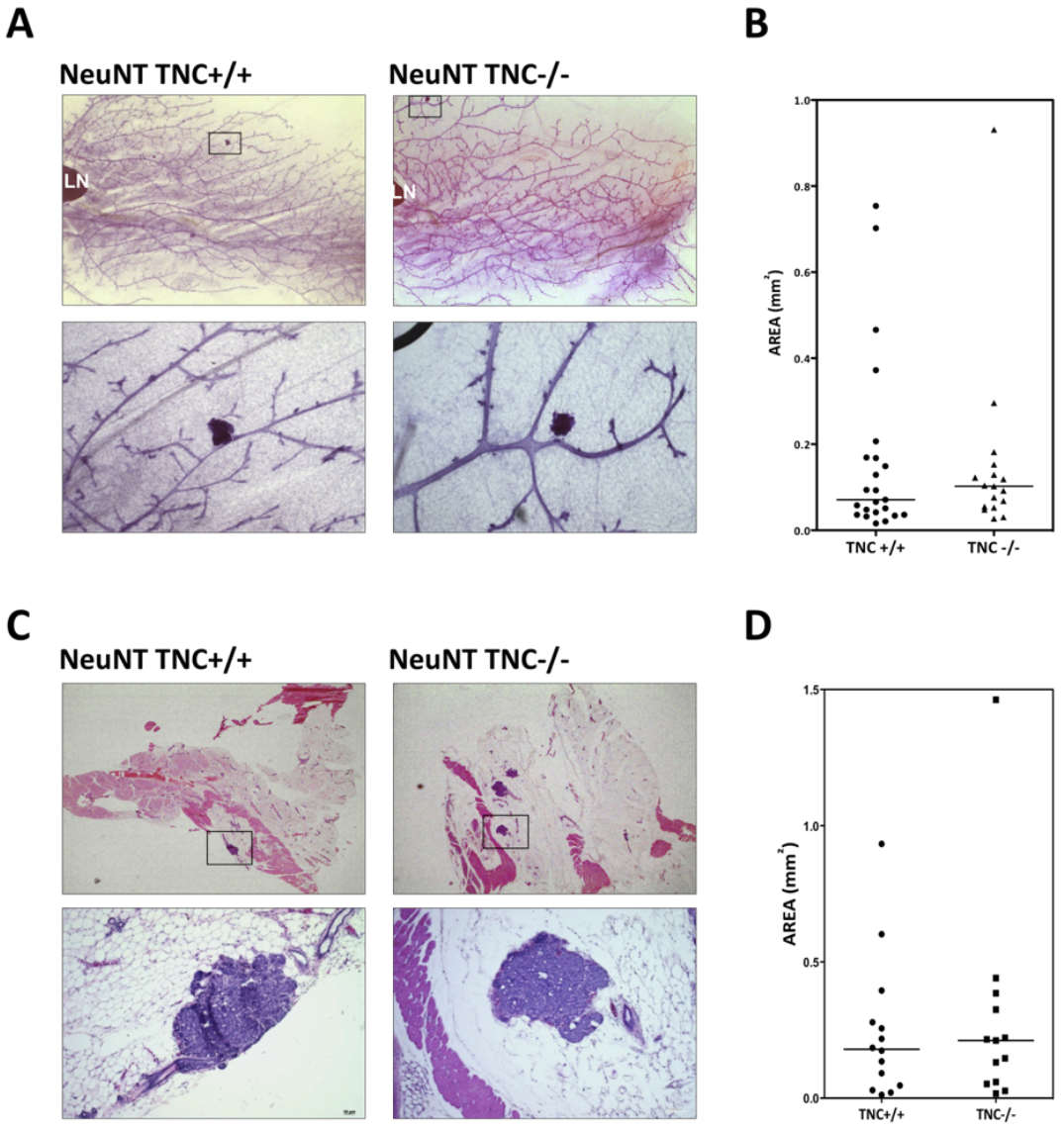


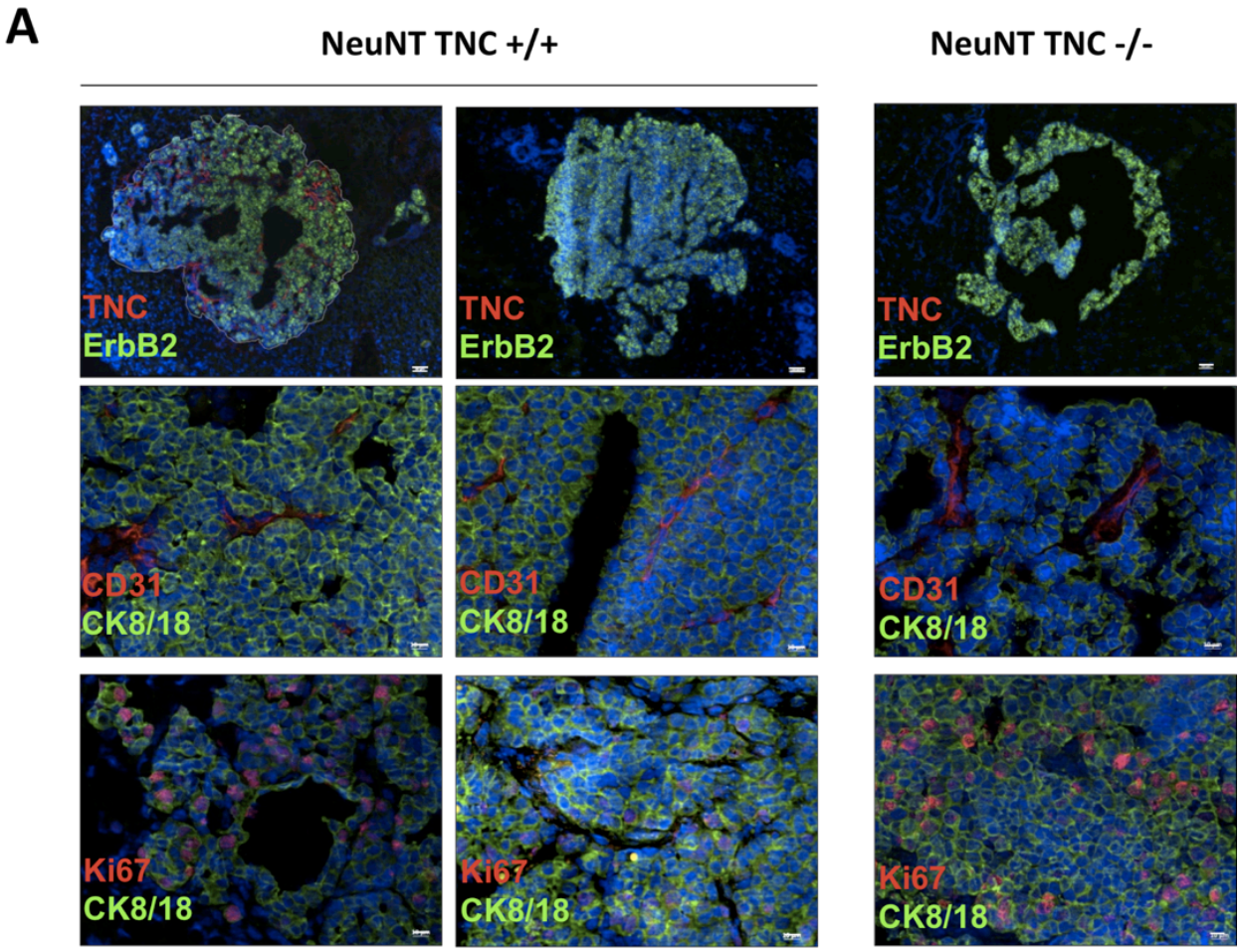
Figure 26. Quantification of lesion size in NeuNT TNC +/+ and NeuNT TNC -/- mammary glands

Analysis of whole mounts **(A,B)** and paraffin embedded tissue **(C,D)** of the 4th, 2nd and 3rd right mammary glands, respectively, of NeuNT TNC +/+ (n = 11) and NeuNT TNC -/- (n = 12) mice. **(A,C)** Overview (upper panels) and closer view (lower panels) are shown. Lymph node (LN) is indicated. The size of the lesions was measured with a grid on a calibrated microscope. The median of the early tumor size is presented. (A) p = 0.6715; (B) p= 0.865, Mann Whitney test.

Trying to understand at which step of early tumor progression TNC potentially is involved, I analyzed its expression in the observed early tumor lesions from NeuNT TNC +/+ mice by IF (**Figure 27A**). Surprisingly I could not detect TNC expression in 3/4 of the lesions. Moreover a detectable TNC expression did not correlate with the size of the lesion (**Figure 27B**). When expressed, TNC formed tracks inside the early tumor lesions (**Figure 27A** top-left panel) and these tracks highly resembled the TNC expression pattern observed in primary breast tumors (endpoint in the previous study) and in lung parenchymal metastases.

Upon further analysis of these early tumor lesions by IF for proliferation and angiogenesis, I observed that already at this early stage cells in all lesions proliferated as expected (**Figure 27A**, lower row) but more surprisingly that CD31 positive EC were detected in all of the lesions including relatively small ones (**Figure 27A**, middle row). Thus, these small nodules represent early tumor lesions. Moreover, they display a heterogeneous TNC expression that does not correlate with lesion size.

To conclude, only a minority (25%) of early (4.5 months) tumor lesions was found with detectable TNC, which did not correlate with lesion size. Still, it is interesting to note that among the TNC positive early lesions was the biggest lesion from the whole series (**Figure 27B**). Despite intense efforts, no differences in the number of these lesions were observed, but only trends. These results are puzzling with regards to the original observation that tumor latency was delayed in TNC -/- animals. Overall, although several technical explanations may exist, one of our hypotheses is that the chosen time point may have been too early (see discussion).



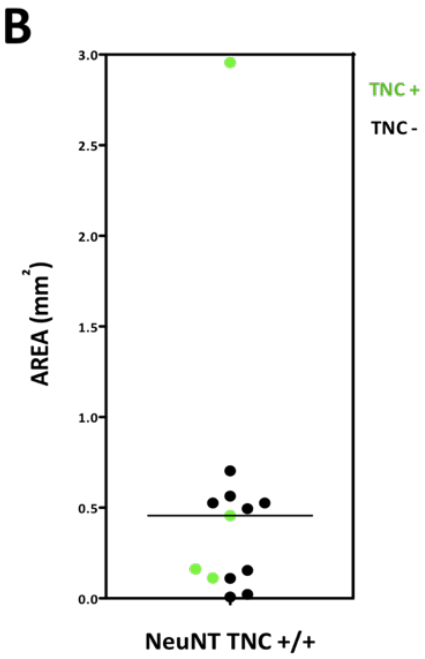


Figure 27. Analysis of TNC expression in early breast tumor lesions from NeuNT TNC +/+ and NeuNT TNC -/- mice

(A) Analysis of 2nd and 3rd left mammary glands from NeuNT TNC +/+ and NeuNT TNC-/- mice. Early tumor lesions were identified on HE-stained adjacent sections. IF showing the expression of TNC, CD31 and Ki67 in early tumor lesions of NeuNT TNC +/+ mice (left and middle columns) and NeuNT TNC-/- mice (right column). Note the presence (left column) and absence (middle column) of TNC in early tumor lesions from NeuNT TNC +/+ mice, respectively. Scale bars upper panels 50 µm, middle and lower panels 10 µm.

(B) Determination of the lesion sizes observed in NeuNT TNC +/+ 2nd and 3rd left mammary glands (N = 6 mice). The presence or absence of TNC was determined by IF (see A). Black dots and green dots represent lesions that do or do not express TNC, respectively. Notice that not all lesions expressed TNC, and that lesion size and CD31 positivity, respectively did not correlate with TNC expression.

3. DEVELOPMENT OF SYNGENEIC BREAST CANCER CELL LINES FOR TUMOR GRAFTING IN IMMUNE COMPETENT CONDITIONS

Genetically engineered mice are powerful tools to study tumor development *in vivo*. However, development of the models and tumor latency can be time-consuming. This is especially true for the NeuNT model for which tumor latency can reach up to 200 days for control mice and more than 300 days for TNC -/- mice, while 3 more months are then further needed for the mice to develop lung metastases. To circumvent this, I generated syngeneic cell lines from PyMT and NeuNT breast tumor tissue. My goal was to use these established cell lines that would grow *in vitro* and also remain tumorigenic when implanted back in syngeneic, fully immuno-competent mice (FVB genetic background). Therefore, PyMT and NeuNT breast tumor tissue was mechanically and enzymatically disaggregated and cells were cultivated for several months to obtain cell lines that reproducibly grew *in vitro*.

3.1 PyMT-derived cell lines

After few passages most of the cells derived from a PyMT tumor (referred to further below as PT248 cell line) showed an epithelial morphology (**Figure 28B**, left panel). IHC analysis demonstrated that they expressed the luminal epithelial cell markers CK8/18 and the oncoprotein PyMT (**Figure 28B**, right panel). To test the tumorigenic capabilities of PT248 cells I implanted them subcutaneously (heterotopic injection) or in the mammary fat pad (orthotopic injection) of FVB immune competent mice. All injected mice developed tumors although tumor growth was heterogeneous (**Figure 28C, D**) and some of them developed lung metastases (55% of mice orthotopically injected, 80% of mice heterotopically injected). Histological analysis of the tumors did not reveal differences between transgenic tumors and their counterparts derived from PT248-grafted cells: both were invasive cribriform carcinomas with differentiated tumor cells that expressed the PyMT oncoprotein (**Figure 28E**). I also collected lung metastasis tissue to establish more aggressive cell lines (i.e. with a higher propensity to give rise to lung metastasis upon subsequent orthotopic grafting). Four different PT248 derived lung-metastatic (PT248-LM) cell lines were established (**Figure 28F**).

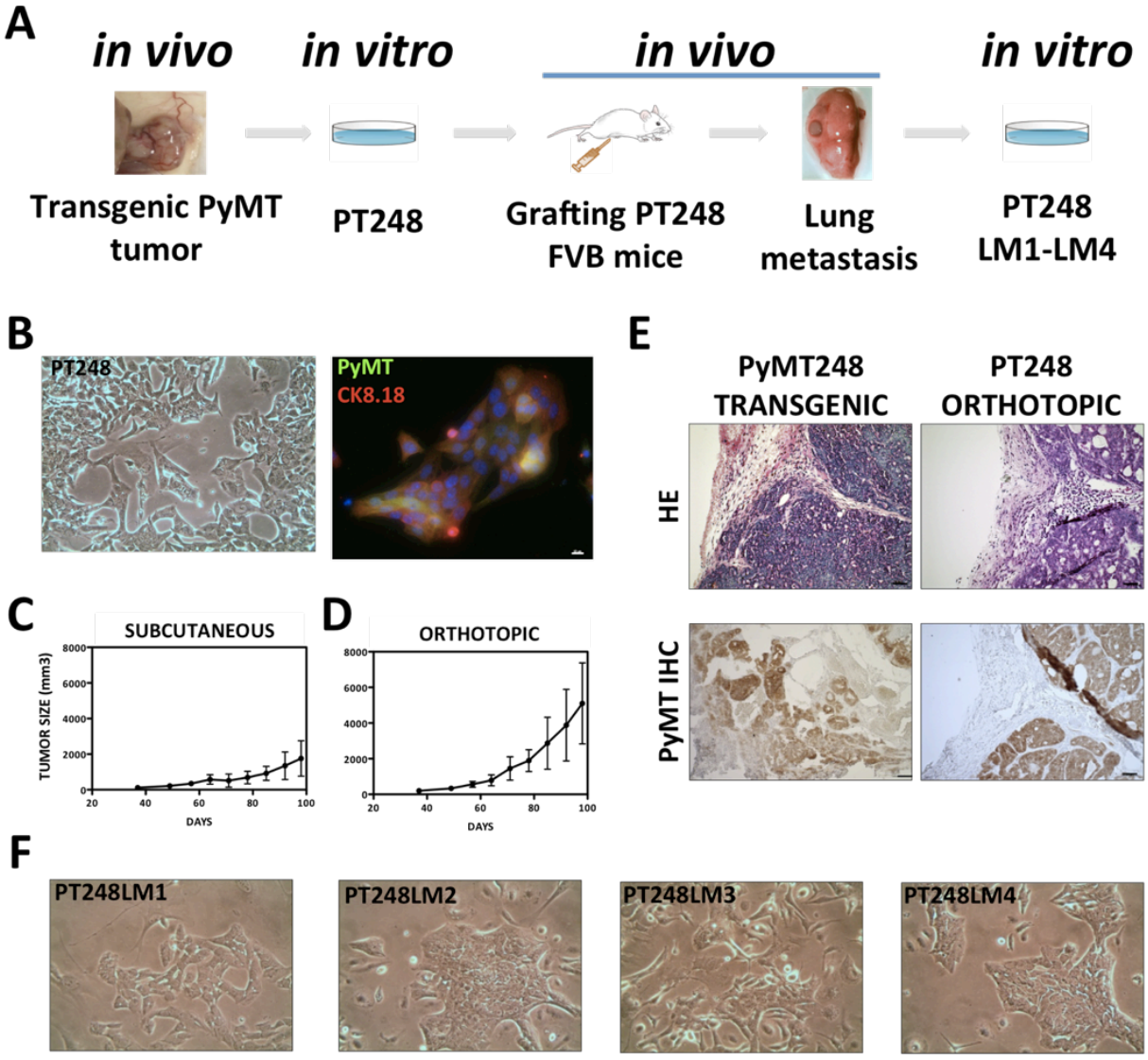


Figure 28. Establishment of PT cell lines and *in vitro* and *in vivo* characterization

(A) Scheme representing the strategy of cell line establishment (PT248) from a primary PyMT tumor and 4 lung metastasis derivatives PTLM1 - PTLM4.

(B) Analysis of PT248 cells grown *in vitro*. After ten passages, most cells represented an epithelial morphology (phase contrast, 20X magnification) which expressed the epithelial cell marker CK8/18 (red) and PyMT (green).

(C-D) Tumor development upon grafting PT248 cells subcutaneously (C, "heterotopic"; n = 6 mice, two injections per mouse) and into the mammary fat pad (D,"orthotopic", ; n = 9 mice, two injections per mouse). Graphs in D show the mean ± 95% CI. Note that all injected tumor cells engrafted and grew in the immune competent host. Tumors grew bigger in the orthotopic than in the heterotopic condition.

(E) Histological comparison by HE and IHC (PyMT) stainings of the transgenic PyMT tumor from which the PT248 cell line was established and an orthotopic tumor that was derived from PT248 cells grafting. Scale bars 50 µm.

(F) Phase contrast images of the PTLM1 - PTLM4 cell lines derived from PT248 lung metastases.

3.2 NeuNT derived cell lines

3.2.1 Establishment of the NT193 cell line and *in vitro* and *in vivo* characterization

A tumor from a transgenic NeuNT mouse (#193) was processed as described before (and in more details in the methods) in order to put cells in culture. After several passages and selective trypsin treatments to remove fibroblast-like cells, most of the cells presented an epithelial morphology (**Figure 29B**, left panel). When NT193 cells grew until confluency they were organized in an epithelial cell compartment and a mesenchymal cell compartment (**Figure 19B**, right panel). Interestingly I also observed the formation of sphere-like aggregates on top of the epithelial monolayer (**Figure 29B**, right panel). By IHC I confirmed that both epithelial and mesenchymal cells expressed the ErbB2 oncoprotein and the luminal cytokeratins 8/18, although the expression of both markers was higher in the epithelial cell compartment. These results show that the shape of NT193 cells is plastic.

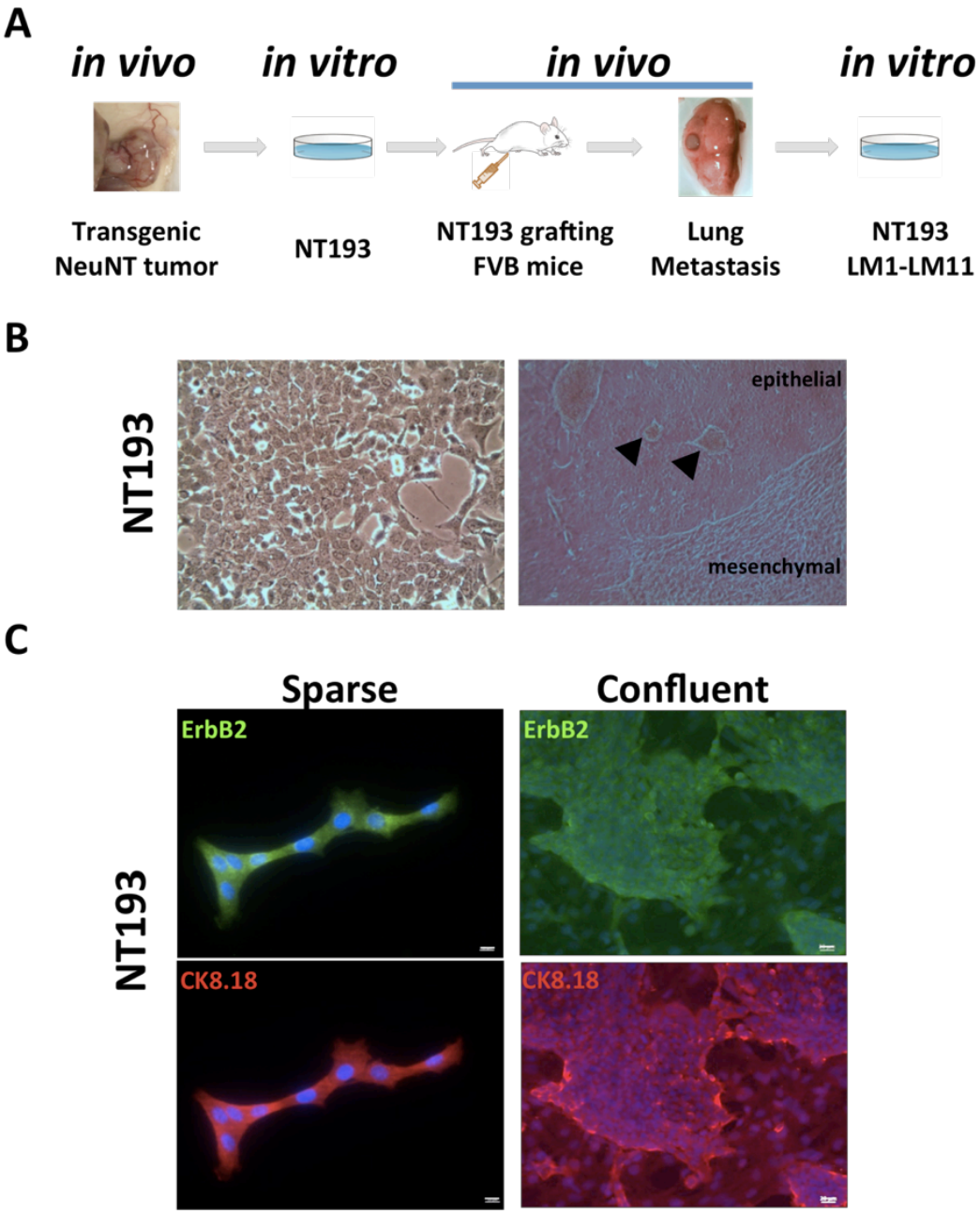


Figure 29. Establishment of the NT193 cell line and *in vitro* characterization

(A) Scheme representing the strategy for cell line establishment (NT193) from a primary NeuNT tumor and 11 lung metastasis derivatives (NT193LM1 – NT193LM11).

(B) Phase contrast pictures of NT193 *in vitro* growing sparsely (left) and confluently (right). Note that when NT193 cells reach high confluence, cells with epithelial and mesenchymal morphologies are observed. Besides sphere-like aggregates (arrowheads) can be observed on top of the epithelial monolayer.

(C) IF analysis of NT193 cells for ErbB2 and CK8/18. Note that even at high confluency NT193 cells with mesenchymal morphology express ErbB2 and CK8/18. Scale bars, sparse cells 10 µm, confluent cells 20µm.

To assess their tumorigenicity, NT193 cells were first implanted either hetero- or ortho-topically in syngeneic (FVB) female mice. Although grafted in fully immune competent female mice, injected cells unexpectedly led to tumor development in 100% of cases (**Figure 30A**). Subcutaneously injected cells generated bigger tumors than orthotopically injected cells (**Figure 30B**). Using conventional histology, I observed that heterotopic and orthotopic tumors derived from the NT193 cell line strikingly phenocopied the parental tumor (which was confirmed by an anatomo-pathologist). All of them were invasive cribriform breast carcinomas with infiltrating stroma and with tumor cells that expressed the ErbB2 oncoprotein and were organized in nests (**Figure 30C**). Interestingly, while subcutaneous tumors did not lead to efficient lung metastasis formation, after 5 months, orthotopically implanted tumor cells metastasized to the lung (overall in 60% of mice initially orthotopically injected with NT193 cells; **Figure 30A**). Both intravascular and parenchymal metastases were observed (**Figure 30D**), demonstrating that NT193 cells recapitulate the metastatic pattern observed in the transgenic animals. Finally, the direct lung metastagenic potential of NT193 cells was also tested upon tail vein injection. Tail-vein injected cells gave rise to lung metastasis in 37% of the injected mice (**Figure 30A**).

A

	SUBCUTANEOUS	ORTHOTOPIC	TAIL VEIN
BREAST TUMOR	100% (10/10 mice)	100% (10/10 mice)	na
LUNG METASTASIS	10% (1/10 mice)	60% (6/10 mice)	37% (1 /8 mice)

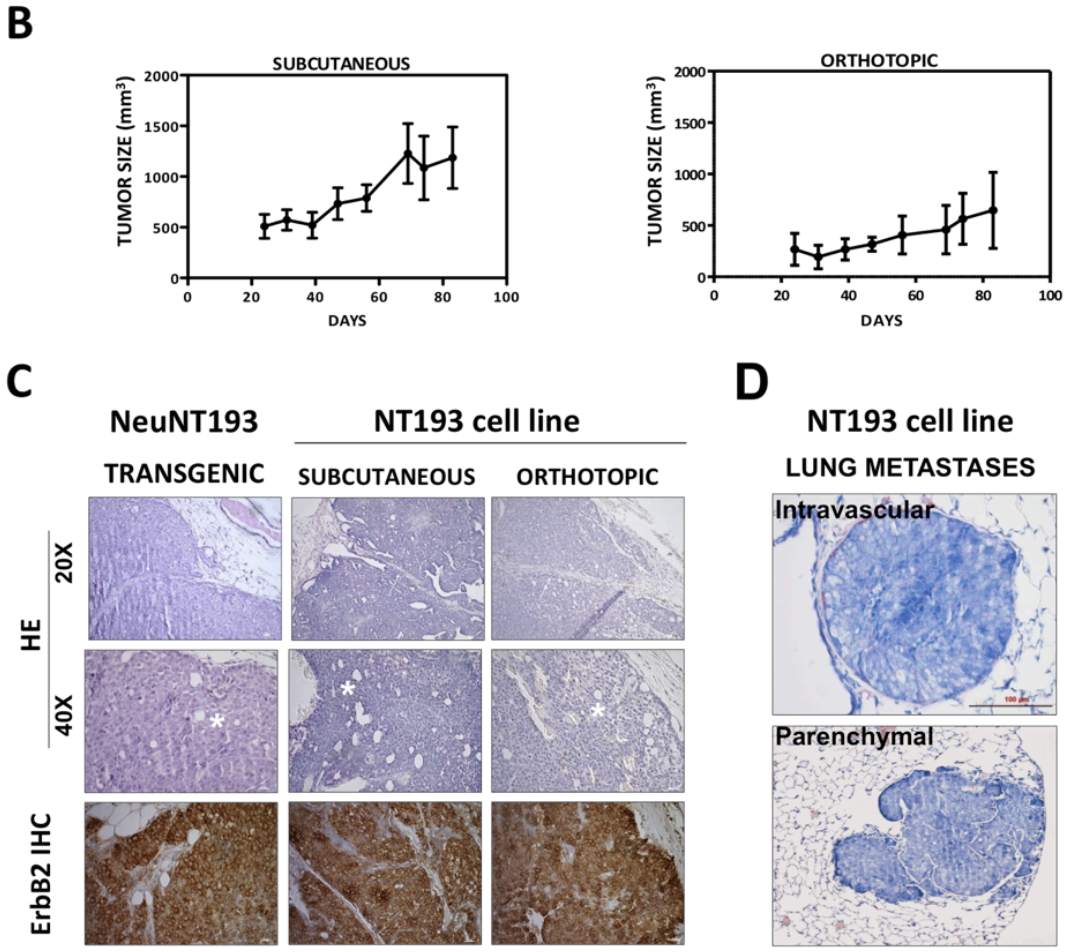


Figure 30. NT193 cell line characterization *in vivo*

(A) Incidence of breast tumors and lung metastasis upon heterotopic (subcutaneous), orthotopic (mammary gland) and tail vein injections of NT193 cells. In each grafting experiment, 10 female mice were injected (on both flanks of a mouse for subcutaneous or orthotopic injections). Mice were sacrificed between 1 and 5.5 months after cell implantation. na, not applicable.

(B) Tumor diameter was measured once per week with a caliper. The graphs represent the mean ± 95% CI. Notice that despite engraftment of the same number of cells, subcutaneously grafted tumor cells gave rise to bigger and faster growing tumors. Lung metastasis was more frequent in orthotopically grafted (60% of mice) conditions than upon subcutaneous grafting (10%).

(C) Histological and IHC comparison of a NeuNT parental transgenic breast tumor with a subcutaneous and orthotopic NT193 cell line derived tumor. Notice that similar to the transgenic tumor, the grafted NT193 derived tumor exhibits stromal infiltration (arrows), a cribriform phenotype (asterisk) and ErbB2 expression. (D) Giemsa stainings of spontaneous intravascular (scale bar, 100µm) and parenchymal (obj. magnification 5X) lung metastases in a mouse upon orthotopic grafting of NT193 cells and tumor growth for 5 months.

3.2.2 Establishment of NT193 cell line derivatives

I have been using the parental NT193 cells to establish several cell line derivatives. First, one of our goals is to obtain highly metastatic cell lines. I therefore established derivatives of the NT193 parental cell line that were selected *in vivo*. To this end, I collected some of the lung metastases obtained from NT193 orthotopic tumors and after NT193 tail vein injections (from the above mentioned experiment, **Figure 31**) to re-derive cells *in vitro* (orthotopic: NT193 LM1-LM7 derivatives; tail vein: NT193 LM8-LM11 derivatives).

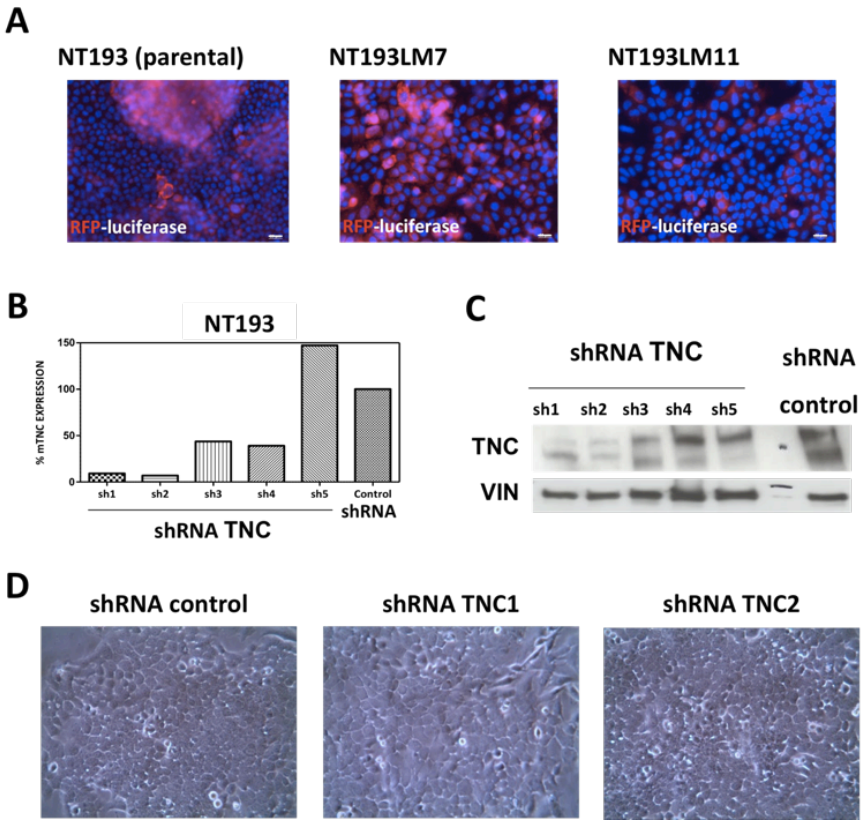


Figure 31. Generation of derivatives from the NT193 parental cell line *in vitro*

(A) NT193 parental cells together with its derivatives NT193LM7 and NT193LM11 cells (previously selected from NT193 lung metastases after injections of NT193 parental cells into FVB mice) were transduced with lentivirus in order to express a fluorescent (RFP, red) tag and the luciferase encoding gene.

(B-C) Test and validation of 5 different shRNA (upon lentiviral-mediated knockdown) in NT193 cells. **(B)** TNC mRNA expression was compared between NT193 control cells and NT193 cells with a stable expression of shRNA 1 – 5 targeting TNC by RT-qPCR. Relative expression to the control is displayed (%). **(C)** Western blot analysis of TNC expression levels in control and shRNA TNC NT193 cells with vinculin (vin) as internal loading control. The shRNA clone 1 (shRNA TNC1) and clone 2 (shRNA TNC2) were found to provide the most efficient knockdown at both RNA and protein levels.

(D) Cell morphology upon TNC knockdown with the 2 independent shRNAs selected from previous analyses (B-C) for further analyses (shTNC1 and shTNC2). Note that no obvious effect was observed concerning the NT193 cellular morphology when the cells were grown in standard adherent conditions.

Second, parental NT193, NT193LM1 and NT193LM11 cells were transduced with lentiviral particles in order to stably express two different tags (from the same vector), RFP and luciferase (**Figure 31A**). Validation of luciferase expression is currently ongoing and will ultimately allow performing non-invasive *in vivo* imaging to monitor and quantify primary tumor growth, dissemination and metastasis formation of these syngeneic grafting models. The expression of the fluorescent tag (RFP in this case; **Figure 31A**) will in particular allow to track disseminating NT193 tumor cells *in vivo* and opens important new avenues of investigation (see discussion).

Third, I found that NT193 cells express TNC *in vitro* (**Figure 31B-C**). Therefore I used a shRNA mediated knockdown strategy to reduce TNC protein levels in these cells (**Figure 31B-C**). The two most efficient shRNAs (shTNC1 and shTNC2; independent TNC sequences targeted) and a control non-target shRNA were selected for further analysis. No obvious difference in cell morphology *in vitro* was detectable between control and TNC knockdown cells (**Figure 31C**). Analysis of the tumor cell autonomous consequences of TNC knockdown in NT193 cells *in vitro* is in progress: several classical cellular traits implicated in tumorigenesis are analyzed (proliferation, apoptosis, migration, invasion). The sphere formation capabilities of NT193 cells are currently being tested as to assess whether TNC plays an important role in these cells similarly to other tumor cell models (124) (150). Finally, TNC knockdown in NT193 cells, used for grafting into a syngeneic TNC -/- FVB host represents novel and powerful tool to address the relative contribution of tumor- and stromal-derived TNC to NeuNT breast tumor progression *in vivo (se below)*.

3.2.3 Assessing tumor versus stromal TNC contribution to NeuNT breast tumor progression

The NT193 cellular model now allows dissecting the relative contribution of tumor- and stromal-derived TNC to breast tumor progression *in vivo*. Of note, this question could not be addressed in the transgenic NeuNT model since TNC was either present (in the control animals, in both tumor and stromal cells) or totally knocked-out (in both tumor and stromal cells).

The selected shControl and TNC knockdown cells were orthotopically implanted in syngeneic (FVB) hosts that were either TNC +/+ or TNC -/-. Altogether this experiment allows addressing the relative contribution of tumor- and stromal-derived TNC to NT193 breast tumor progression *in vivo* (**Figure 28A**). Although this experiment was only very recently terminated and requires validation of the stability of TNC knockdowns *in vivo,* several interesting observations were already made. No major differences were observed concerning both tumor frequency (**Figure 28B**) and tumor burden after 3 months of growth (**Figure 28C**). This latter result again supports the previous conclusion (from the study using the transgenic model) that TNC is dispensable for NeuNT primary tumor growth. Metastasis formation was (so far) only quantified during dissections by systematic and careful examination of all lung lobes under a stereoscope (**Figure 28D**). This analysis of surface macrometastases revealed that i) control cells metastasized in both TNC +/+ and TNC -/- hosts but seem to form less macrometastases in the TNC -/- hosts (**Figure 28E**). This shows that stromal TNC promotes the formation of these macrometastases. TNC knockdown cells seemed impaired in macrometastases formation, but to various degrees (**Figure 28E**). NT193 shTNC1 cells never formed lung macrometastases (either in TNC +/+ or TNC -/- hosts), suggesting that tumor-derived TNC is also critical for the formation of macrometastases. NT193shTNC2 cells formed 1 macrometastasis each in two TNC +/+ mice (**Figure 28D-E**), a similar proportion of mice as compared to shcontrol cells in TNC +/+ hosts, but who formed more macrometastasis (4 and 10 in these mice, **Figure 28D-E**). As previously mentioned, before drawing more conclusions from this experiment, i) the stability of the TNC knockdown *in vivo* needs to be validated and ii) metastatic burden needs to be quantified on tissue sections. Both are ongoing.

A

Tumor *versus* stromal TNC contribution to NeuNT breast tumor progression		NT193 tumor cells	
		shControl	shTNC
Syngeneic murine host	TNC +/+	Tumor: TNC+ Stromal: TNC+	Tumor: TNC- Stromal: TNC+
	TNC -/-	Tumor: TNC+ Stromal: TNC-	Tumor: TNC- Stromal: TNC-

B

Tumor Frequency		NT193 tumor cells		
		shControl	shTNC1	shTNC2
Syngeneic murine host	TNC +/+	63% N = 8, n = 10/16	50% N = 7, n = 7/14	100% N = 7, n = 14/14
	TNC -/-	93% N = 7, n = 13/14	72% N = 7, n = 10/14	100% N = 4, n = 8/8

C

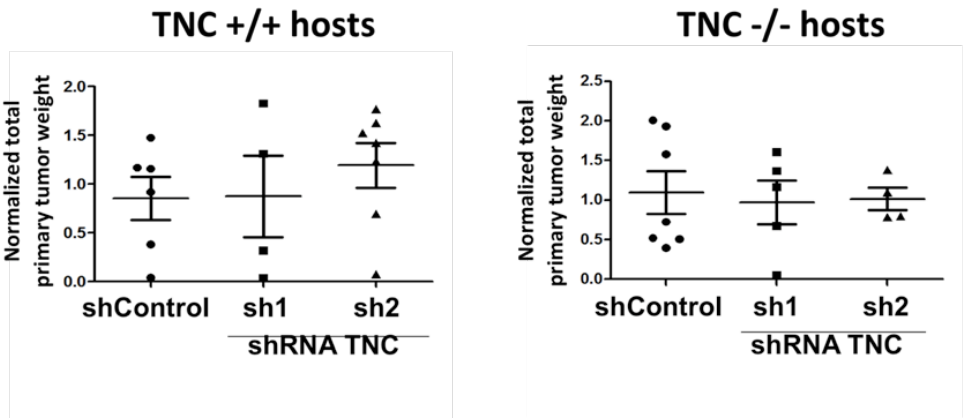
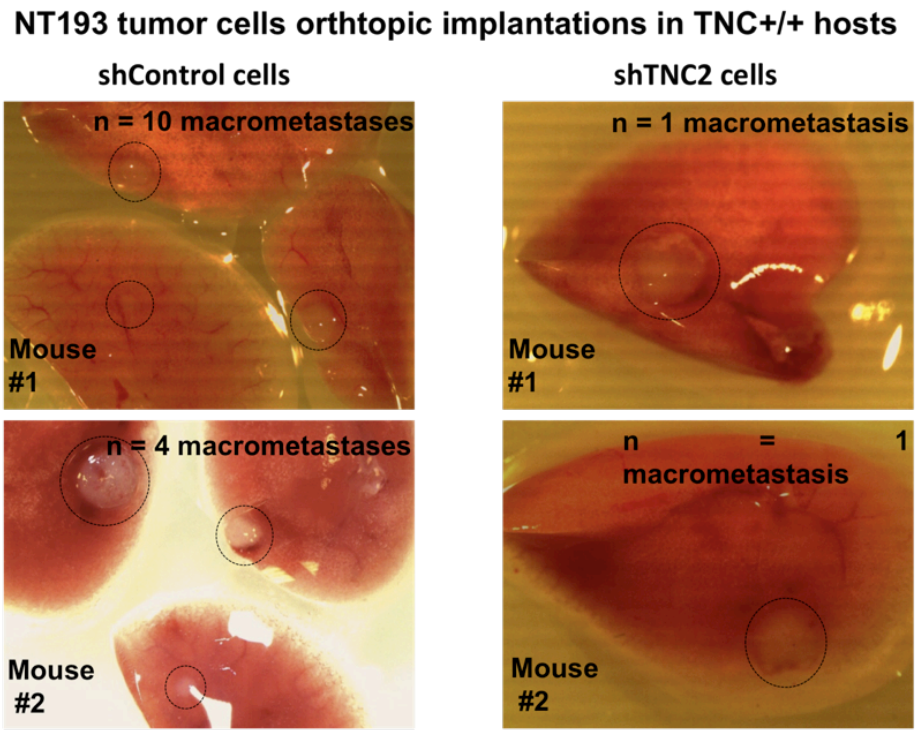


Figure 32. Analysis of the relative contribution of tumor and stromal-derived TNC to NT193 breast tumor progression in vivo

(A) Table recapitulating the possible tumor and stromal TNC contribution in each experimental condition. For each condition, the possible expression/functional contribution of the tumor (+: possible; - : none). For simplification purposes, the conditions where NT193 shTNC cells are used are represented as "TNC negative" (TNC-). However note that TNC expression was silenced *in vitro* by selecting cells stably expressing shRNAs but may still express residual TNC protein levels. On the contrary, in TNC -/- hosts, stromal cells can not express TNC and therefore stromal contribution is fully ablated.

(B, C) NT193 breast primary tumor take rates and tumor burden 3 months after grafting for NT193 shcontrol, sh TNC1 and shTNC2 cells implanted in TNC+/+ and TNC-/- FVB hosts. Note in particular that no significant differences were observed in primary tumor burden after 3 months.

D



E

Lung Metastasis		NT193 tumor cells		
		shControl	shTNC1	shTNC2
Syngeneic murine host	TNC +/+	25% (N =2/8) n = 14 macromets	0% (N = 0/7)	29% (N = 2/7) n = 2 macromets
	TNC -/-	29% (N = 2/7) n = 2 macromets	0% (N = 0/7)	0% (N = 0/4)

Figure 33 continued. Analysis of the relative contribution of tumor and stromal-derived TNC to NT193 breast tumor progression in vivo

(D) Representative macroscopic pictures of the macrometastases observed on the lung surface of TNC+/+ FVB mice upon dissection. NT193 cells (shcontrol cells, left column; shTNC2 right column) were orthotopically implanted and tumors allowed growing for 3 months. Obvious macrometastases were observed for 2 mice (out of 8 dissected) injected with NT193 shcontrol cells (10 and 4 metastases in each mouse) and for 2 mice (out of 7) injected with NT193 shTNC2 cells (1 and 1 macrometastasis in each mouse). None of the TNC +/+ mice injected with NT193 shTNC1 cells developed lung macrometastases.

(E) Summary on frequencies and cumulated metastases number per condition. Interestingly, these data altogether suggest that both tumor- and stromal-cell derived TNC may contribute to the outgrowth of lung macrometastases.

SUMMARY

Altogether I had used two established transgenic breast tumor models with induction of stochastic multifocal adenocarcinomas induced by two different oncogenes (PyMT and NeuNT) to address the roles of TNC in tumor onset and progression. Therefore I had analyzed mice with transgenic overexpression of TNC and a TNCKO and compared results to mice with TNC wildtype levels. Overexpression of TNC in both TNC transgenic tumor models did not appear to reach high overexpression of TNC, which could explain the absence of a phenotype. In the PyMT model I confirmed that the absence of TNC (now analyzed in a pure background) did not have an impact on tumor growth and lung metastasis.

In the NeuNT model I found that the absence of TNC increased tumor latency. Preliminary results suggest that in the absence of TNC onset of dysplasia is delayed which surprisingly is not reflected in tumor burden that was similar between tumor mice expressing or lacking TNC which raises the question of potential compensatory mechanisms in the absence of TNC **(Figure 33A)**. Metastasis in NeuNT mice is characterized by intravascular emboli and a parenchymal phenotype. TNC is expressed in both subtypes. Expression of TNC around the metatastic emboli suggests a potential function in tumor cell survival and extravasation into the lung parenchyma. This possibility is supported by the observation that apoptosis is increased in the tumor emboli in the absence of TNC. This may explain the reduced size of tumor emboli in the absence of TNC. Tumor cells within the tumor emboli can enter the lung parenchyma to give rise of macrometastasis. In the absence of TNC less parenchymal metastases were observed. Thus my results suggest that TNC promotes tumor cell survival in the tumor emboli and their entry into the lung parenchyma **(Figure 33B)**.

Finally I had established cell lines from PyMT and NeuNT mice and showed that they induce tumors and lung metastasis in a syngeneic host with an intact immune system**.** The NT193 cell line had been engineered for TNCKD and was used for grafting into a host lacking TNC. In this ongoing study I observed that tumor engraftment was not affected by TNC since this was similar irrespective of the presence or absence of host and stromal TNC. This study revealed that one of the TNCKD cell lines was impaired in macrometastasis induction. Together the newly established grafting model applying immune competent hosts with defined TNC expression levels and syngeneic breast cancer cell line NT193 with defined TNC

levels is a valuable tool to address the mechanisms underlying the role of TNC in promoting lung metastasis. In collaboration within the team the NT193 cell line was used to address the impact of a ErbB2 targeting peptide (MTP-ErbB2) on tumor cell survival demonstrating that this cell line provides also a valuable tool for drug discovery research (see discussion).

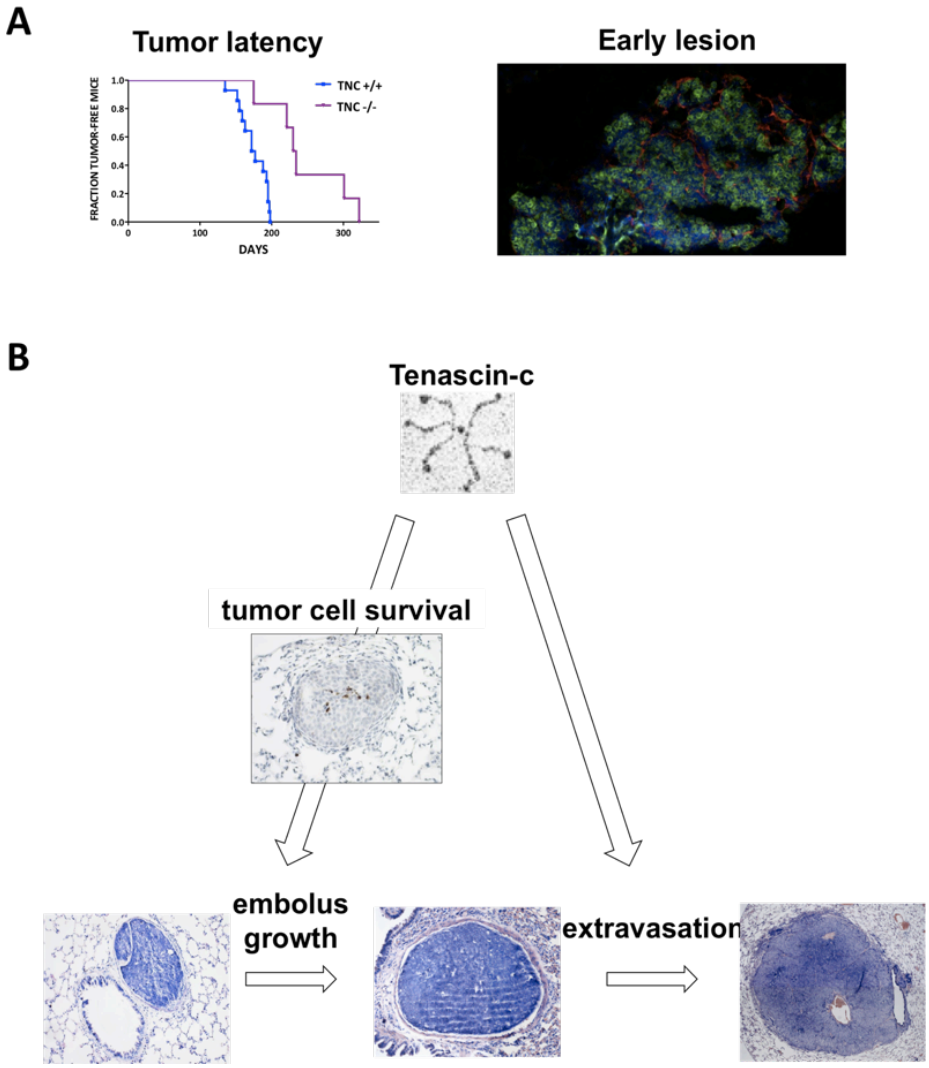


Figure 33. Summary of the major results on the roles of TNC in NeuNT breast tumor progression

(A) TNC promotes early step(s) during NeuNT breast tumor establishment/progression at the primary site.

(B) TNC promotes late steps during NeuNT breast tumor progression by enhancing lung metastatic colonization.

DISCUSSION

The tumor microenvironment plays a crucial role in tumor progression. There is evidence of TNC, highly expressed in malignant breast tumors, to promote lung metastasis formation in breast cancer patients (40) (130). Although important insights into the underlying molecular and cellular mechanisms have been obtained (101) (124), a comprehensive view about the roles of TNC in breast cancer onset and progression is missing in the literature. Given that TNC regulates immune cell behavior (140), and most studies have been done in xenograft models lacking an intact immune system, it is evident that important functions of TNC might have been missed. Therefore, in this study we used two well-characterized murine breast cancer models with relevance to the human disease, the MMTV-PyMT and MMTV-NeuNT models. The goal was to compare tumorigenenic parameters in mice with no TNC (TNCKO), wildtype and TNC overexpression. The following results will be discussed in detail:

1.- Overexpression of transgenic TNC does not appear to reach levels that induce a phenotype in the PyMT and NeuNT model.

2.- Knock out **of** TNC in PyMT mice did not result in a difference in tumorigenesis and metastasis even in a pure genetic background confirming that TNC is dispensable in the PyMT context.

3.- NeuNT/TNCKO mice experienced a delay in the appearance of palpable tumors. We observed a tendency for a decrease in the tumor burden in the absence of TNC. However this difference is not statistically significant, suggesting that TNC is dispensable in growth of primary NeuNT tumors.

4.- NeuNT/TNCKO mice experienced reduced lung metastasis. TNC promotes survival of tumor cells in the metastatic emboli and entry of tumor cells into the parenchymal lung tissue.

5.- The NT193 grafting model is a valuable tool for addressing the mechanisms of breast cancer lung metastasis formation and associated thromboembolism in immune competent conditions, and for drug discovery research.

1. No impact of transgenic TNC on breast tumorigenesis in MMTV-PyMT and MMTV-NeuNT mice

In the past, double transgenic mice have been successfully used to analyze the consequences of overexpression of a protein in cancer and particularly in breast cancer (151) (56) (152). In these studies

mice that express the gene of interest (mostly signaling receptors or soluble factors such as TGF-β1 (143) or VEGF (152)) in the mammary gland using the MMTV promoter were bred with mice that develop tumors due to expression of an oncogene in the mammary epithelium (e.g. MMTV-PyMT or MMTV-NeuNT). Using this strategy it was demonstrated that transgenic TGF-β1 promotes lung metastasis in a Neu-driven murine breast cancer model (143) and that transgenic VEGF promotes metastasis in breast cancer-prone NeuYD -mice (152).

Our goal was to obtain double transgenic animals that express transgenic TNC in the mammary gland of females together with the oncogenes PyMT and NeuNT, respectively. Therefore, single transgenic MMTV-TNC mice that had been generated previously in the laboratory were bred with either MMTV-PyMT or MMTV-NeuNT mice and double transgenic mice were obtained and analyzed.

Four MMTV-TNC founder lines 7, 8, 9, 10 passed the transgene to their progeny and were used for generating mice for analysis. Offspring of these founders expressed the transgene at mRNA level in the mammary gland of virgin female mice as well as in breast tumors and metastatic lungs of NeuNT/mTNC mice (founder 9). However, TNC mRNA expression levels in NeuNT/mTNC tumors do not exceed those expressed in NeuNT tumors.

Previous studies in the laboratory revealed that the TNC transgene is translated into an apparently full length TNC protein in transfected cultured cells. This, however, does not ensure that the transgenic TNC protein is also well expressed in the tumors *in vivo.* Indeed, preliminary results from a few analyzed NeuNT tumors indicate that TNC expression levels in TNC transgenic tumors do not exceed the expression levels present in tumors with wildtype TNC. This observation needs to be substantiated by analyzing more tumor samples. Since the MMTV promoter can be induced by lactogenic hormones, we hypothesized that low TNC protein levels is due to a poor activation of the promoter in tissue of virgin mice (5). Insufficient stimulation of the MMTV promoter does apparently not apply to the oncogenes PyMT and NeuNT that induce transformation of the mammary epithelium even under virgin conditions. But these are powerful oncogenes that may exhibit a biological response already upon induction of low levels of expression. In contrast a structural protein such as TNC may only elicit a phenotype when overexpressed at very high levels. Thus, it is possible that higher transgenic TNC levels would have been reached if tumorigenic females had undergone pregnancy before tumor development.

In our laboratory a new model of inducible TNC expression has been generated (O. Lefebvre, C. Spenle, G. Orend). If when using this model TNC levels exceed those levels present in tumor mice with wildtype TNC,

this inducible TNC expression model can be use to analyze the effect of the overexpression of TNC in breast tumor formation and lung metastasis colonization.

In another TNC transgenic tumor model generated in the laboratory, the Rip1Tag2 model, already moderate expression of the transgenic mRNA (human sequence) resulted in a clear tumorigenesis-promoting phenotype (Saupe et al., in preparation). Arguably, a moderate additional expression of TNC from the transgene eventually has a biological impact since ECM molecules are usually stable and can accumulate over time in the microenvironment. It is possible that this scenario applies to the Rip1Tag2 model but not to the two used breast cancer models. Finally, TNC is already highly expressed in the tumorigenic mammary gland of PyMT and NeuNT mice (wildtype TNC) and thus it may be impossible to reach even higher levels of TNC to induce an additional effect.

2. Effect of the absence of TNC on breast tumor progression

Some studies show that expression of TNC correlates with a poor prognosis (114) (116) (113) (104). In addition, *in vitro* and *in vivo* studies show that TNC promotes proliferation, invasion, migration, angiogenesis and lung metastasis formation (140) (99) suggesting that TNC promotes tumor progression through one or multiple of these mechanisms. Consequently, we expected that the absence of TNC would negatively affect breast tumor progression. Our results show that whereas the absence of TNC does not alter tumor progression in the PyMT breast cancer mouse model, it delays tumor onset and reduces lung metastasis in the NeuNT-driven breast cancer model.

2.1 No impact of the lack of TNC on tumorigenesis and metastasis in a pure background of PyMT mice

Talts and collaborators (1999) had already investigated whether the absence of TNC in PyMT mice had an impact on tumorigenesis and lung metastasis. But these studies were performed in a mixed background, which might have increased the variability of the model affecting the conclusions. Therefore we repeated the experiment in a pure FVB background. We observed no difference in tumor burden and tumor characteristics between PyMT mice expressing TNC and those not expressing it. In mice of both genotypes there was also no detectable difference in lung metastasis colonization. Thus, our results confirm the previous study (131).

The logical conclusion of these observations is that TNC does not play an essential role in breast tumor progression. However, this conclusion counters previous observations carry on in other models, namely that TNC affects breast cancer progression and particularly lung metastasis formation (40) (130)(124).

An alternative explanation is that other proteins expressed in the PyMT mouse model drive the processes carried on by TNC in other models. In line with this hypothesis it was shown that in PyMT mice the absence of periostin (POSTN) severely impairs lung metastasis formation. The function of POSTN was linked to the binding of Wnt ligands within the metastatic niche and thereby activation of Wnt signaling in PyMT metastasis-initiating cells (134). Since TNC can also activate Wnt signaling in other models (153) (124) (Saupe et al., in preparation), it is possible that in the PyMT model, in which Wnt signaling can be activated by POSTN, TNC is dispensable. Therefore, further research may analyze the effect of the absence of both POSTN and TNC in PyMT-driven breast cancer progression. It has been proposed that POSTN and TNC could cooperate in breast cancer lung metastasis (132), which potentially involves cooperative Wnt activation. The cooperative action of TNC and POSTN could be tested using the newly established grafting model with cells and hosts that exhibit defined levels of both ECM molecules.

Another possibility is that the absence of TNC in the KO mouse model might be compensated resulting in no obvious difference in tumorigenesis. Potential compensation in the absence of TNC is discussed amongst the community for non-tumorigenic TNCKO mice since the time when TNCKO mice were born alive and exhibited no gross abnormalities (154) (155). Since TNC is highly expressed during development and highly conserved among mammals, it was anticipated that the absence of TNC would cause a lethal phenotype. This is not the case. Thus, as yet poorly understood compensatory mechanisms might be at play (156). Interestingly, PyMT/TNCKO tumor tissue was more heavily infiltrated by macrophages than tissue from PyMT tumors with TNC wildtype. Also the organization of the tumors was different (131). Thus, in the future it remains to be seen whether macrophages have contributed to the absence of difference in tumor malignancy in PyMT/TNCKO mice. TNC is part of an ECM network and there might be feedback regulatory mechanisms if one prominent ECM component such as TNC is missing. In an expression profiling experiment comparing angiogenic and non-angiogenic pancreatic islets from Rip1Tag2 mice, an AngioSwitch signature was identified. In this signature, several ECM molecules including TNC, were highly induced (Langlois, Saupe, Rupp, Hussenet and Orend, in preparation). Upon comparison of the AngioSwitch signature between wildtype TNC and TNCKO mice it was observed that the angiogenic switch was largely reduced in the absence of TNC (Saupe et al, *in preparation*). Moreover, expression of many of the genes in the AngioSwitch signature were several fold elevated in angiogenic islets lacking the TNC protein. These results suggest molecular compensation in the absence of TNC. Understanding the exact mechanisms of the process, however, requires future research.

2.2 Increased tumor latency, similar tumor burden and, reduced lung metastasis in the absence of TNC in NeuNT tumor mice

In contrast to the PyMT model where no impact of TNC on tumorigenesis and lung metastasis was seen, in the NeuNT model TNC appears to promote tumor initiation and metastatic lung colonization.

In this study it was found that the absence of TNC delays tumor detection from a median tumor latency of 174 days to more than 230 days without an obvious effect on tumor burden when assessed after 3 months of tumor detection. Due to breeding issues the mouse cohort available for analysis was small with only 6 NeuNT TNCKO mice. Whereas NeuNT mice with one TNC allele did not show a difference to mice with two TNC alleles, presumably because TNC levels were not significantly different (c.f. **figure 8**), mice lacking TNC experienced a significant delay in tumor detection.

Generally, a delayed onset on breast tumors in mouse models indicates the need of additional genetic events to develop malignancy (151), although the delay could also be caused by a reduction in the expression levels of the oncogene (41). Additionally the numbers of early (non palpable) lesions, as well as the growth rate of these lesions into palpable tumors, affect the tumor latency.

2.2.1 Delayed onset of dysplasia in NeuNT/TNCKO mice

To determine a potential impact of TNC on early events in tumorigenesis before tumor detection, tumor tissue of NeuNT control and NeuNT/TNCKO mice was prepared at 4.5 months of age. At this age, a tumor had never been detected in this study. Moreover, at 3 months of age small tumor lesions had been detected in 2 out of 3 analyzed NeuNT mice (whole mount preparation of the mammary gland), suggesting that 4.5 months was an appropriate time point to detect early tumor lesions in most of the NeuNT mammary glands.

In control mice as well as in TNCKO mice, we observed that early NeuNT breast tumor lesions are highly heterogeneous in frequency and size, and probably in differentiation status. This heterogeneity demonstrates the development of early tumor lesions in a non-uniform fashion presumably caused by a heterogeneous expression of the oncogene. In our work we observed mammary glands without lesion, with only one, and with more than 3 lesions. Moreover, not only the number of lesions per mammary gland was very heterogeneous but also the size of the lesions varied: it went from small lesions of < 0.01 mm^2 to almost 1 mm^2 in diameter. Finally, we observed relatively large *in situ*-like lesions, which contrasted with the very small but already locally invasive lesions (**Figure 24**). Our analysis showed that the TNC genotype does not statistically significantly affect the number or the size of early tumor lesions.

This model with intrinsic heterogeneity complicates the analysis of very early transformation events. To address whether TNC potentially has an impact on the earliest tumorigenic events, an arbitrarily defined sub-classification of the early tumor lesions was done by using the number of lesions per mouse as a classification criterion. This analysis revealed that while the mammary glands of NeuNT control mice displayed a comparable distribution of 30% class I (no lesion), 40% class II (1 - 3 lesions) and 30% class III lesions (more than 3 lesions per mammary gland), the relative distribution of the lesion subclasses was different in mammary glands of NeuNT/TNCKO mice (with 60% class I, 30% class II, and 10% class III lesions). Although these results are not statistically significant, the observed tendency in tumorigenic TNCKO mice of 2-fold more lesion-free glands and 3-fold less glands with more than 3 lesions might be biologically significant. This observation presumes that TNC impacts on events that trigger dysplasia, which is also reflected in increased tumor latency in the absence of TNC.

To determine when TNC is expressed during dysplasia, TNC expression was analyzed using tissue staining. Remarkably, only 30% (4/13) of the analyzed early tumor lesions (N = 6 NeuNT female mice, n = 13 early tumor lesions) expressed TNC at 4.5 months of age. Moreover, one mammary gland simultaneously showed lesions expressing TNC and lacking TNC. Preliminary analysis of the morphology of the early tumor lesions of TNC +/+ mice, suggest that the lesions expressing TNC are more invasive. These observations need to be further evaluated with specific markers (e.g. cytokeratins 5 and 6) in more samples. A lack of TNC expression in non-invasive lesions would be in agreement with previous work demonstrating that TNC is poorly/not at all expressed in non-invasive breast tumors (157) (102).

More surprising was the observation that the expression of TNC apparently does not correlate with the size of the tumor lesions. Although there are no data published that correlate early lesion size with TNC expression, there are two publications that documented a correlation between TNC expression and the size of the breast tumors (114) (116).

One possible explanation for the above results is that NeuNT mice develop two types of early tumor lesions. One type requires TNC for growth, while the other type´s growth is independent of TNC. The appearance of a tumor lesion depends on a balance between tumor cell proliferation and cell death. Therefore, future research needs to determine whether TNC expression in early tumor lesions correlates with cell survival and/or proliferation.

Angiogenesis is important for tumor growth (14). We observed that very small lesions were already vascularized without any evident correlation with TNC expression. Again, more samples need to be analyzed to substantiate this observation. In the future it remains to be seen whether angiogenesis plays a role in early lesion growth and metastasis in the NeuNT model (see below).

In summary, although analysis of early tumor lesions did not reveal a statistically significant difference in numbers of lesions between NeuNT mice with wildtype TNC and a TNCKO, this study showed that TNC might promote dysplasia onset. Our study also allowed defining a window for future analysis of the transformed mammary gland (4.5 months) to address in more detail TNC´s role in the onset of tumorigenesis.

2.2.2 No impact of TNC on tumor growth: endpoint analysis

Our results showed that (i) the absence of TNC in NeuNT mice increases tumor latency (as determined by tumor palpation), which suggests that TNC promotes tumor onset. (ii) Analysis of tumor lesions at the age of 4.5 months further suggests a potential role of TNC in promoting the appearance of dysplastic lesions, which indicate a role of TNC on tumor onset. (iii) At the end point of analysis a (3 months after tumor detection) no statistically significant difference in total tumor mass was observed between NeuNT mice with wildtype TNC and a TNCKO, suggesting that TNC does not promote expansion of early lesions into tumors, and/or that TNC is not required for promoting the growth of established tumors.

Whether TNC expression levels impact the size of breast tumors remains unclear in the literature. Whereas some studies in breast cancer patients found that high TNC expression correlates with bigger tumors (114) (116), other reports that do not confirm this correlation (108) (111). Using xenograft models, four different studies using melanoma (150) or breast cancer cell lines (40) (124) (41) tested the tumor-initiating and metastatic potential of tumor cells with wildtype and reduced (TNCKD) TNC expression. All studies found that lowered TNC levels in the tumor cells correlated with reduced lung metastasis. The reduced TNC expression (TNCKD), however, had a heterogeneous effect on primary tumor growth. Whereas no impact on growth of the primary tumor was seen in the majority of previous studies, Calvo and collaborators (using the MDAMB435 cell line) observed that a TNCKD interfered with primary tumor growth.

A potential explanation for the lack of an impact of TNC on tumor growth in the xenograft models could be compensation by the host. However, the study carried out by O'Connell and collaborators (2011) does not support this possibility (101). This group analyzed the effect of the absence of stromal TNC on breast tumor and lung metastasis formation upon orthotopic implantation of 4T1 cells into a syngeneic host lacking TNC (TNCKO in Balb/c mice). They did not observe a difference in tumor size when comparing breast tumors in a TNC wildtype and TNCKO host. It is worth noting that in this study the authors

reported the size of the primary tumor 24 days after tumor cell implantation with no reference to growth of the tumors at earlier time points. Since in our hands 4T1 cells do not express TNC *in vitro* it is likely that this experimental model (4T1 cell implantation in TNCKO mice) mimics the TNCKO condition in the NeuNT model where we neither observe a size difference in comparison to tumor mice with wildtype TNC.

A major gap in our analyses regards the lack of information about the tumor growth (the size of the tumor at different time points) neither in PyMT nor in NeuNT mice. The analysis in multifocal transgenic models is because the first tumor detected rapidly fuses with other tumors present in the same or adjacent mammary glands. As a result, we do not know if the similarities between the tumor burden of control and TNCKO mice are 1) because TNC does not participate in tumor growth or 2) because TNC favours tumor growth only during the first phases of tumor development, becoming dispensable after. A potential solution to circumvent this problem is the application of the NT193 grafting model (see below).

Newly formed blood vessels support tumor growth. Therefore we investigated tumor angiogenesis by measuring CD31 signals as readout for endothelial cells. Results showed no difference detectable in tumors of NeuNT mice with wildtype TNC and TNCKO. Thus, in this model TNC does not have an impact on endothelial cell numbers and thus presumably on the tumor vasculature.

2.2.3 Reduced lung metastasis in NeuNT/TNCKO mice

The NeuNT model exhibits intravascular and parenchymal metastasis. We found that the majority of the lung metastases in NeuNT mice were intravascular. This observation is in accordance with several ErbB2-induced breast cancer mouse models (143) (65) (76) (142). These metastatic emboli are mostly composed of epithelial cells (CK8/18 positive) that express the oncogene ErbB2, thus demonstrating their tumorigenic nature. Moreover many cells within the metastastic emboli proliferate at similar rates than in the parenchymal metastases. It was shown that tumor cells at the periphery of the emboli can undergo EMT triggered by TGFβ signaling which drives tumor cells to enter the parenchymal tissue and establish macrometastasis in the lung (143). Thus metastatic emboli can be considered as a transitory stage towards the formation of parenchymal metastasis that eventually will lead to organ failure and patient death. This is also supported by the relative distribution of intravascular and parenchymal metastasis at earlier time points. Whereas at 1-4 waftd (weeks after first tumor detection) mice only displayed intravascular metastasis, at 6-9 and 10-17 waftd only 60% and 50% of all metastases were intravascular and 40% and 50% were parenchymal, respectively.

Tumor emboli are not artifacts of breast cancer mouse models. The presence of tumor cell emboli within the vasculature have been recurrently observed in cancer patients (145). Such tumor cell emboli have been reported both in the vasculature near the primary tumor site (158) and at distant sites such as the lung, suggesting that tumor emboli can play an role in tumor cell dissemination and lung metastasis (32) (33). Accordingly, the presence of obvious peritumoral vascular emboli has been correlated with a bad prognosis and the formation of lung metastasis in cancer patients including breast cancer patients (158) (146) (147) (148). Retrospective analyses of large cohorts indicated different incidences of tumor emboli (from 0.9% to 26%). Interestingly the presence of tumor emboli is particularly frequent in breast cancer patients (159) (145). It has been previously reported that several pathological manifestations are due to the presence of the tumor emboli; these pathologies include pulmonary hypertension, myocardial hypertrophy, and death caused by respiratory distress (159). Additionally it is thought that pulmonary tumor emboli can be a source of cancer cells in the bronchovascular area (159).

We determined the expression of TNC in the lung metastasis, and observed that TNC is expressed in both metastasis subtypes. Whereas TNC was organized in fibrillar tracks in the parenchymal metastasis, TNC was present at the periphery of the intravascular metastasis surrounding the embolus. This coat can also contain ECM molecules such as FN and LM. Since TNC and FN are frequently co-expressed in tumor tissue, in particular around newly formed blood vessels (160), it is possible that both molecules play a collaborative role in metastasis of NeuNT mice.

What could be the role of TNC in these metastatic emboli? Does TNC provide shielding towards immune cells, a mechanical coat to resist shear stress in the circulation, or does TNC serve as a bridge to the lung endothelium to facilitate tumor cell breaching into the lung parenchyma?

To tackle these questions the cellular composition of the emboli was analyzed. By immunostaining S100A4-positive mesenchymal cells (Mts1), EC (CD31) and platelets were found to be present at the periphery but rarely inside the emboli. Since S100A4-positive mesenchymal cells express TNC (101) it is possible that these cells are a source of TNC also in this model. They may also be involved in recruitment of EC as seen in other cancers. During the quantification of intravascular and parenchymal metastases using CD31 immunostaining as marker for blood vessels, a layer of endothelial cells was found to surround the tumor emboli.

Endothelialization of tumor emboli has previously been reported by Sugino and collaborators (33) (32) in their analysis of tissue from lungs and breast tumors derived from a breast cancer xenograft mouse

model. Endothelialized tumor emboli were even present in the vicinity of the primary breast tumor (33) (32). Oshima and collaborators (2004) using the MMTV-NeuYD (a less aggressive Neu-driven breast cancer mouse model) showed that overexpression of VEGF promoted the endothelialization of the tumor emboli and the earlier formation of intravascular lung metastases. The authors proposed that tumor cells might leave the primary tumor together with the adjacent endothelium that ends up surrounding the embolus.

Moreover platelets ($CD41^+$) are present in a high number of tumor emboli localized as TNC in the periphery of the embolus (colocalization had not been addressed yet). In this scenario, it is possible that platelets adhere to and activate upon the direct contact with TNC as had been observed in *in vitro* experiments (161) .

Recently, Labelle and collaborators (2012) showed that a co-culture of cancer cells with platelets induced secretion of TGF-β1 by the platelets that activated TGFβ/Smad signaling in the cancer cells, inducing EMT and promoting extravasation into the lung parenchyma (23). Analyzing their available gene expression data (23), we observed that activated platelets also had an effect on TNC expression. In a potential scenario platelets are recruited to the metastatic embolus where they secret TNC and/or are attracted by TNC at the periphery of the emboli. Tumor cells in contact with the platelets may undergo an EMT that facilitates tumor cell entry into the lung parenchyma. If TNC is important in attraction of platelets there should be less platelets present in the tumor emboli of TNCKO tumor mice. This should be investigated in tissue of NeuNT/TNCKO mice. The newly established NT193 grafting model can also be used to address a potential complicity of TNC and platelets in breast cancer lung metastasis (see below).

To address the question whether TNC has an impact on NeuNT induced lung metastasis, lung metastases in NeuNT control and TNCKO, mice were determined 3 months after detection of the primary tumor. This analysis did not reveal a statistically significant difference when quantifying all metastases. However, most of the analyzed NeuNT TNCKO mice (5/6) contained few metastases compared with the control group (median TNCKO = 2.5, median control =6). One of the TNCKO mice developed the highest number of metastases observed (44 metastases). Since in this model the number of lung metastasis is highly variable (from none to almost 30 metastases/lobe in control animals) more samples need to be investigated. It is interesting to note that this TNCKO mouse that developed the most lung metastases exhibited the longest latency (322 days), suggesting that despite the absence of an overt tumor, tumor cells disseminated and formed metastasis in the lung. In this particular case it was noticed that the

metastases were very small, suggesting that TNC supports the metastasis growth in the lung parenchyma. This result is similar to the study of O'Connell (101).

Whereas the overall number of metastases was not different in the absence of TNC, the size of the lung metastases was lowered. This applies in particular to the size of the intravascular metastases. Several studies in immune compromised mice using melanoma (150) or breast cancer cell lines (40)(41)(124)(101), showed that the absence of TNC correlates with a reduced metastatic lung colonization. Now we confirmed this observation in a stochastic immune competent model where we saw significantly smaller intravascular emboli in the absence of TNC. This may be linked to the observation that in the absence of TNC more tumor cells die in the emboli. We further observed that the transition of the emboli into the parenchyma is impaired in the absence of TNC. These results thus suggest that TNC promotes tumor cell survival in the metastatic emboli, which promotes their growth and their potential to enter the lung parenchyma.

Previously, two research groups showed that TNC increases the survival of breast cancer cells in lung metastasis thus promoting lung metastasis colonization. O'Connell and collaborators (2011) using a model in which the main source of TNC are S100A4^{+} cells, demonstrated that depletion of these cells reduced metastasis colonization by promoting cell death. The authors proposed that this survival effect is mediated by TNC (101). Oskarsson and collaborators (2011) reported that cancer cell-derived TNC (124) increases cell survival in lung metastasis without affecting cell proliferation. The authors showed that in their xenografting model using among others MDAMB231-derivatives, the apoptosis protective effect of TNC is linked to TNC activation of Notch (MSI1) and Wnt (LGR5) signaling (124).

In summary, in the absence of TNC the number of intravascular metastases and size of metastatic emboli were reduced presumably due to an enhanced apoptosis. Tumor cells from the intravascular metastases can penetrate the lung parenchymal tissue upon EMT. Since metastatic emboli represent a step towards parenchymal metastasis, a reduced number of metastastic emboli could explain a lower number of parenchymal metastasis in the absence of TNC. TNC is expressed as a "coat" around the intravascular metastatic emboli presumably playing several roles, namely 1) shielding against shear stress and immune cell attacks, and 2) providing a microenvironment that supports EMT and angiogenesis presumably through attraction of platelets and S100A4-positive mesenchymal stromal cells. The reduced parenchymal metastasis phenotype in the TNCKO could be explained by a role of TNC in promoting cell survival in the intravascular emboli and in facilitating EMT in the intravascular metastatic emboli.

2.3 Differences in the role of TNC in PyMT- versus NeuNT-driven breast cancer progression.

It remains unclear the reason why PyMT and NeuNT breast cancer mouse models display so different effects in the absence of TNC. Both models generate luminal adenocarcinomas (142)(65) with histological similarities (162) (and observations of the pathologist Marie-Pierre Chenard) that metastasize to the lung. More important, previous work characterizing the expression profiles of several breast cancer mouse models determined that PyMT and Neu-driven breast tumors display similar molecular patterns (59). Finally, the oncogenes that produce tumors in both mouse models (ErbB2 in the NeuNT model and the polyoma virus middle T antigen in the PyMT model) encode transmembrane proteins with associated kinase activity that can activate MAPK and PI3K pathways (68)(84). Actually, some researchers consider PyMT as "a molecular surrogate" for ErbB2 (163)(164).

In spite of these similarities, several differences between these models were observed. At a genetic level, reports show significant differences in the frequencies of change at certain loci comparing the copy-number aberrations between ErbB2- and PyMT-based tumors (165). Additionally, at the molecular level Mohamed Bentires-Alj and Benjamin G. Neel demonstrated that the absence of protein-tyrosine phosphatase 1B importantly delay the tumor detection in NeuNT mouse model without having any influence in PyMT-driven breast tumor progression (166). This single example shows that, although similar in several aspects, particularities between NeuNT and PyMT breast cancer models should not been neglected.

Both PyMT and NeuNT oncoproteins activate similar intracellular pathways (please refer to the introduction). Therefore, it will be interesting to know if TNC affect differently tumor-cell autonomous mechanisms and or stromal cell-tumor cell crosstalks. Future research may address these possibilities through in vitro analysis.

3. Syngeneic breast cancer cell lines

With the goal of saving time we generated two syngeneic breast cancer cell lines (PT248 and NT193) that grow *in vitro* and that generate breast tumors and lung metastasis in vivo.

The establishment of breast cancer cell lines proved more complex than expected, for it took us several months before we could obtain the described PT248 and NT193 cell lines (and around 5 months before being able to test the tumorigenecity of the cells *in vivo*). The establishment of a cell line of NeuNT transgenic breast tumors was particularly difficult. Even if we collected cells from 6 tumors coming from 4 different animals, we succeeded with only one of them

Our data showed that both PT248 and NT193 cell lines produce tumors in 100% of the immune competent mice when injected subcutaneously or in the mammary gland. Histological comparison of transgenic (PyMT or NeuNT) *versus* syngeneic (PT248 or NT193) breast tumors does not reveal any difference between them. Impressively, MP Chenard, an expert anatomopathologist in breast cancer from the Hautepierre Hospital (Strasbourg), could not distinguish tumors derived from the transgenic and the grafting model when examining conventionally stained tumor tissue from these models in blind conditions. Additionally these cells maintain the expression of the oncogenes of their parental transgenic model (PyMT or ErbB2) after growth *in vitro* and *in vivo*.

Moreover, both cell lines form spontaneous lung metastases and interestingly we observe intravascular and parenchymal metastases in some of the mice implanted with NT193 cells showing altogether that these cells are able to reproduce all the breast cancer progression process, as observed in the transgenic models. We are interested in obtaining cell lines able to form lung metastases; hence we recovered macrometastases from the syngeneic injected animals (PT248 and NT193) and grew them *in vitro*. In addition we transduced some of the NT193 lung metastasis derived (LM) cell lines along with the NT193 parental cell line to generate RFP-luciferase expressing derivatives. *In vitro* and *in vivo* analysis of the LM lung-derived cell lines (from either PT248 or NT193) as well as their RFP-luciferase derivatives is still in progress.

More importantly, NT193 cells express TNC (**Figure 31**). This allowed using shRNA-mediated technology to analyze the consequences of TNC knockdown on tumor cell autonomous mechanisms *in vitro* (proliferation, invasion, migration, sphere formation assays), and *in vivo* (to analyze the effect of the absence of cell specific TNC in breast tumor progression, see below).

In addition, these cell lines will represent a valuable tool for the scientific community as a novel, syngeneic and spontaneously metastatic cellular model for ErbB2-driven breast cancer cells. In fact, the members of our team Alexia Arpel and Dominique Bagnard already used the NT193 parental cell line. The NT193 cell line was used to analyze the consequences of a novel peptidic strategy and was instrumental in providing biological validation of the approach (Arpel et al., in prep; co-author; **Figure 34**).

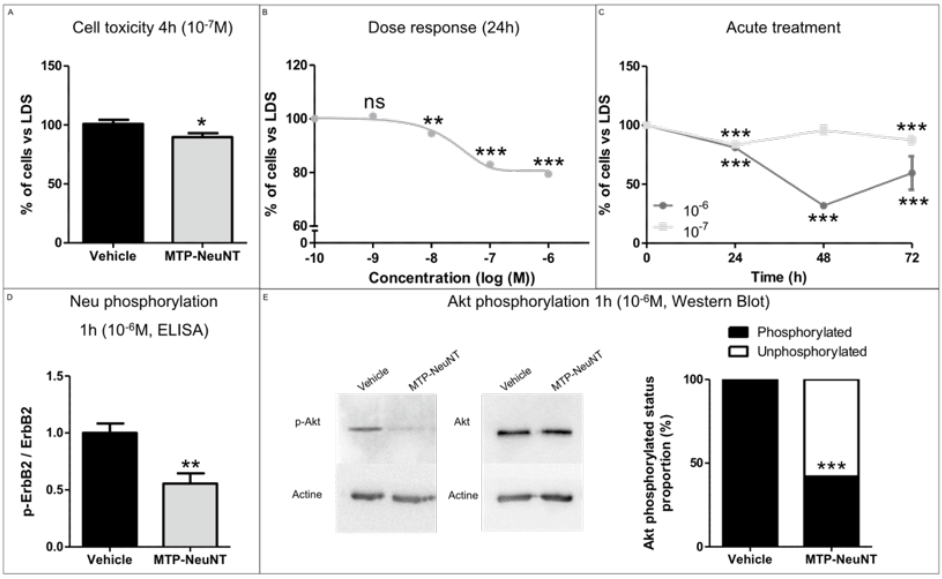


Figure 34. Effect of MTP-NeuNT in vitro on cell proliferation and Neu signaling

Courtesy of A. Arpel and D. Bagnard (figure taken from Arpel et al., manuscript in preparation in the laboratory , I. Velazquez-Quesada co-author)

Characterization of MTP-NeuNT impact on NeuNT193 cell line (results are expressed in percentage of cells versus the vehicle (LDS)). A. Effect of MTP-NeuNT on cell toxicity. Cells were treated 4h with MTP-NeuNT or vehicle at 10^{-7}M before measuring cell survival, MTP-NeuNT had a mild but significant -10% toxicity effect on the tumor cells. B: Effect of MTP-NeuNT on cell proliferation. Cells were treated with increasing doses of MTP-NeuNT (ranging from 10^{-9}M to 10^{-6}M) for 24h before measuring cell proliferation. Dose-response analysis reveals that a significant reduction of cell proliferation is obtained with 10^{-8}M of the peptide reaching a maximal efficacy at 10^{-6}M (-21% at 10^{-6}M versus vehicle). C: Long lasting effect of MTP-NeuNT on NeuNT cells. Cell proliferation was measured at 24h, 48h, 72h after an acute treatment of MTP-NeuNT at day one. This long lasting effect is dose dependent indeed with 10^{-7}M of the MTP-NeuNT peptide a slight reduction observed over time, whereas with 10^{-6}M cell proliferation is still decreased by 41% at 72h. D, E. Inhibition of Neu signaling, D: Decrease of Neu receptor phosphorylation. Treatment of cells with MTP-NeuNT for 1h at 10^{-6}M reveled a significant 45% reduction in Neu phosphorylation. E: Decrease of p-Akt phosphorylation. After 1h treatment of MTP-NeuNT at 10^{-6}M on NeuNT cells Akt phosphorylation is decreased by 58%.

In summary, grafts of NT193 cells in immune competent mice induce breast tumors with a similar phenotype as the genetic MMTV-NeuNT model: similar tissue anatomy, lung metastasis with embolus and parenchymal phenotypes. Preliminary results suggest that tumor growth is similar in the absence of stromal- and tumor- derived TNC, but that lung metastasis is reduced. If validated, these data confirm an important role of TNC in lung metastasis and suggesting that both tumor and stromal-derived TNC contribute to the formation of parenchymal lung macrometastases. Altogether the NT193 grafting model is a valuable tool for addressing the mechanisms of lung metastasis formation and thromboembolism (a complication often observed in breast cancer patients) in immune competent conditions. Finally, the NT193 grafting model also is a valuable tool for drug discovery research.

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lo que quiera que seas luce pequeña estrella"

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WR: Sometimes, just one second." (LC)

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¿Para qué *entonces* sirve la utopía?

Sirve para esto: para caminar”

Fernando Birri

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