PRONGF/NGF AND NERVE INFILTRATION IN PROSTATE AND BREAST CANCER

JAY PUNDAVELA

(BMedSci, MSc)

Thesis submitted in fulfillment of the requirements for obtaining the degree of

DOCTOR OF PHILOSOPHY

School of Biomedical Sciences and Pharmacy

University of Newcastle

February 2015

"Accept the view that nothing in nature is useless, even from the human point of view (with the necessary restrictions of time and place). Even in the rare instance where it may not be possible to use particular scientific breakthroughs for our comfort and benefit, there is one positive benefit – the noble satisfaction of our curiosity and the incomparable gratification and feeling of power that accompany the solving of a difficult problem."

−Santiago Ramón y Cajal,

TABLE OF CONTENTS

TABLE OF CONTENTS

DECLARATIONS PART A

TESTIMONY OF ORIGINALITY

*The thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository**, subject to the provisions of the Copyright Act 1968.*

*(**Unless an Embargo has been approved for a determined period)*

TESTIMONY OF AUTHORSHIP

I hereby certify that the work embodied in this thesis contains a published paper/s/scholarly work of which I am a joint author. I have included as part of the thesis a written statements for each published work, endorsed by my supervisor, attesting to my contribution to the joint publication/s/scholarly work.

THESIS BY PUBLICATION

I hereby certify that this thesis is in the form of series of published papers of which I am a joint author. I have included as part of the thesis written statement from each co-author, endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications.

Signature: …….. *Date:* 09/02/2015

Jay Pundavela *dd /mm / yyyy*

DECLARATIONS PART B

DECLARATION OF CONTRIBUTION TO PUBLISHED WORK

I the undersigned corresponding author of the following publications:

Bradshaw RA, Pundavela J, Biarc J, Chalkley RJ, Burlingame AL, Hondermarck H, **NGF and ProNGF: Regulation of neuronal and neoplastic responses through receptor signaling**. Adv Biol Regul, 2014, S2212-4926(14)00062-1, [Epub ahead of print]

Pundavela J, Demont Y, Lincz LF, Roselli S, Thorne RF, Bond D, Bradshaw RA, Walker MM, Hondermarck H, **ProNGF correlates with Gleason score and is a potential driver of nerveinfiltration in prostate cancer**, Am J Pathol, 2014, 184(12): 3156-62

Pundavela J, Roselli S, Faulkner S, Attia J, Scott RJ, Forbes JF, Bradshaw RA, Walker MM, Jobling P, Hondermarck H, **Nerve fibers Infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer**, Mol Oncology, 2015 [in Review]

Jobling P, Pundavela J, Oliveira SMR, Roselli S, Walker MM, Hondermarck H, **Nerve-cancer cell crosstalk: a novel promoter of tumor progression**, Can Res, 2014 [Accepted]

Authorize the inclusion of these works and declare that Research Higher Degree candidate Jay Pundavela contributed to the paper/publication. Outlined below are the items that the candidate have contributed towards the fulfillment of the papers:

- *Conducted and designed most of the experiments*
- *Critically analyzed and interpreted the results*
- *Prepared and organized the figures*
- *Contributed in drafting and conceptualizing the manuscripts*
- *Contributed in formatting initial and revised versions of the manuscripts*

Signature: …….. *Date: 09/02/2015*

Hubert Hondermarck *dd /mm / yyyy*

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisors Prof Hubert Hondermarck and Prof Rodney Scott for their unwavering support, mentorship, guidance and encouragement enabling me to complete this thesis. Also, special acknowledgement to Dr Phillip Jobling and Prof Ralph Bradshaw for their expert inputs on neuroanatomy and neurotrophic growth factors respectively.

I extend my gratitude to the members of our research team present during my candidature that have given me all the help, advice and friendship, which made this a great doctoral journey. Including Sheridan Keene, Camila de Oliveira and Sam Faulkner who have been of great assistance in my day-to-day experiments. I thank Dr. Severine Roselli of her expert advise on planning and designing of my experiments.

Also I would like to offer my appreciation to a cohort of people in the Department of Medical Biochemistry for their assistance, enthusiasm and friendship that made the laboratory an enjoyable workplace.

Greatest gratitude to my family for their unconditional support and encouragement in my PhD. Especially to my mum, Menchie Lowe and siblings, Justine and Jefri Pundavela. Thank you all for always being understanding and compassionate in times of need.

To my dearest wife, Mengna Chi, without you this PhD wouldn't have been possible. I appreciate your determined full support and trust in my abilities to succeed. I am looking forward and committed hand-in-hand with you into our future.

DEDICATIONS

I would like to dedicate my efforts laid in the success of this thesis to my families back in the Philippines who were devastated by the great typhoon Haiyan that happened during my candidature. Most especially to my sweet grandmother Flocerfina Radam, Uncle and Aunts, cousins and my father Prisco Pundavela. The courage and strengths they showed confronted by the challenges were my inspiration.

LIST OF PUBLICATIONS INCLUDED AS PART OF THE THESIS

CONTAINED IN:

CHAPTER 2 *(THESIS BACKGROUND)*

Bradshaw RA, Pundavela J, Biarc J, Chalkley RJ, Burlingame AL, Hondermarck H, **NGF and ProNGF: Regulation of neuronal and neoplastic responses through receptor signaling**. Adv Biol Regul, 2014, S2212-4926(14)00062-1, [Epub ahead of print]

CHAPTER 3

Pundavela J, Demont Y, Lincz LF, Roselli S, Thorne RF, Bond D, Bradshaw RA, Walker MM, Hondermarck H, **ProNGF correlates with Gleason score and is a potential driver of nerveinfiltration in prostate cancer**, Am J Pathol, 2014, 184(12): 3156-62

CHAPTER 4

Pundavela J, Roselli S, Faulkner S, Attia J, Scott RJ, Forbes JF, Bradshaw RA, Walker MM, Jobling P, Hondermarck H, **Nerve fibers Infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer**, Mol Oncology, 2015 [Accepted].

CHAPTER 5 *(FINAL DISCUSSION OF THIS THESIS)*

Jobling P, Pundavela J, Oliveira SMR, Roselli S, Walker MM, Hondermarck H, **Nerve-cancer cell crosstalk: a novel promoter of tumor progression**, Can Res, 2014 [Accepted].

LIST OF ABBREVIATIONS

0-9

50B11	DRG neuronal cell line
6-OHDOPA	6-hydroxydopamine

A

ATCC	American Type Culture Collection
AIC	Akaike information criterion
ANOVA	analysis of variance

B

BDNF	brain-derived neurotrophic factor
BPH	benign prostate hyperplasia
BCA	bicinchoninic acid assay
BIC	bayesian information criterion

C

CNS	central nervous system

D

DMEM	Dulbecco's modified eagle medium
DAB	3,3' – diaminobenzidine
DCIS	ductal carcinoma *in situ*
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion

E

ERK	extracellular signal-regulated kinase
EGF	epidermal growth factor
ER	estrogen receptor
EDTA	ethylenediaminetetraacetic acid

F

FAK	focal adhesion kinase
FCS	fetal calf serum
FGF	fibroblast growth factor

G

GABA	gamma-aminobutyric acid
G-CSF	granulocyte-colony stimulating factor
GP110	glycoprotein 110
GTP	guanosine triphosphate
GlcNAc	N-Acetylglucosamine

H

HMEC	human mammary epithelial cells
HER2	human epidermal growth factor receptor 2
H_2O_2	hydrogen peroxide

I

IHC	immunohistochemistry
IR-Dye	infrared dye
IgG	immunoglobulin G
IDC	invasive ductal carcinoma
ILC	invasive lobular carcinoma

K

kDa	kilodalton

M

MCF-7	Michigan Cancer Foundation -7
MDA-MB-231	M.D. Anderson - metastatic breast
MAPK	mitogen-activated protein kinase
mM	millimolar
mm	millimeter
µL	microliter
MS	mass spectrometry
MS/MS	tandem MS

N

NGF	nerve growth factor
NT3	neurotrophin 3
NT4	neurotrophin 4
NFκB	nuclear factor kappa B
NTSR3	neurotensin receptor 3
NaCl	sodium chloride

P

proNGF	precursor of NGF
$p75^{NTR}$	p75 neurotrophin receptor
PDGF	plate-derived growth factor
PI3K	phosphoinositide-3-kinase
PGP9.5	protein gene product 9.5
PC12	pheochromocytoma cells
PCR	polymerase chain reaction
PNI	perineural invasion
PLCγ	phosphoinositide phospholipase C gamma

Q

qRT-PCR	quantitative reverse transcription polymerase chain reaction

R

RPMI	Roswell Park Memorial Institute media
RTK	receptor tyrosine kinase

S

SAS	Statistical Analysis System
STAT	Signal Transducer and Activator of Transcription
SILAC	stable isotope labeling of amino acids in culture
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SORT	sortilin

T

TMA	tissue microarray
TNBC	triple negative breast cancer
TrkA	tropomyosin receptor kinase A
TNFR1	tumor necrosis factor receptor 1
TRADD	TNFR1-associated death domain protein
TBS	Tris-buffered saline
Tris-HCl	Tris-hydrochloric acid
TiO_2	titanium dioxide
TCGA	The Cancer Genome Atlas

V

VEGF	vascular endothelial cell growth factor

ABSTRACT

Nerve fibre infiltration has recently been demonstrated as paramount to the tumor microenvironment and essential to cancer progression. However, it has not been clearly elucidated what attracts neuronal fibres into tumors. A plausible explanation is the overexpression of neurotrophic factors, such as nerve growth factor (NGF) or its precursor proNGF. These proteins are distinctively involved in neuronal survival/apoptosis through interactions with specific membrane receptors such as TrkA, $p75^{NTR}$ and sortilin.

This thesis aims to show whether proNGF/NGF overexpression drives nerve infiltration in prostate and breast cancer. What has been showed herein is that the overexpression of proNGF in a cohort of 104 prostate cancer cases, observed using immunohistochemistry has a direct correlation with the aggressiveness of prostate cancer (t_B= 0.51). A 60-kilodalton proNGF was detected by western blotting of prostate cancer cells, whereas the proteolytically processed NGF was not detected. In addition, we performed an *in vitro* co-culture of prostate cancer cells with neuronal cells and demonstrated the neurotrophic effect of prostate cancer cells to stimulate axonogenesis via the secretion of proNGF. Furthermore, in breast cancer we have shown nerve fibre infiltration by immunohistochemistry using the neuronal marker protein gene product (PGP) 9.5. Nerve infiltration was found to be associated with NGF expression and lymph node invasion. Secreted NGF was detected by dot blot analysis of conditioned medium from breast cancer cells. Interestingly, *in vitro* co-culture assays demonstrated that NGF secreted from breast cancer cells stimulated neurite outgrowth of neuronal cells, and that this effect could be inhibited by using an anti-NGF blocking antibody, thus supporting the neurotrophic potential of these cells.

In conclusion, we have shown that proNGF is a driver of nerve infiltration in prostate cancer and that a similar phenomenon occurs in breast cancer via NGF. This study suggests new avenues for inhibiting prostate and breast cancer growth and metastasis by inhibiting axonogenesis via the targeting of proNGF and NGF.

CHAPTER 1 THESIS OVERVIEW

1.1 INTRODUCTION

Recent breakthroughs in prostate (Magnon et al., 2012) and gastric cancer (Zhao et al., 2014) have shown that nerves infiltrate the tumor microenvironment and are active in triggering cancer progression and invasion through the release of neurotransmitters, such as catecholamines and acetylcholine that would bind to tumor membrane receptors. Importantly, the presence of nerve fibres in such tumors is associated with the propensity of the disease to be aggressive and features a poor prognosis. Despite these recent landmark studies, the mechanisms are yet to be demonstrated. We raised the hypothesis that nerve growth factor (NGF) and its precursor protein, proNGF, may be responsible for stimulating neuronal outgrowth in tumors.

1.2 AIMS

The aims of this thesis were to investigate the levels of proNGF/NGF in prostate and breast cancer and determine whether they are associated with the presence of nerve fibres and aggressiveness in these malignancies.

1.3 ORGANIZATION OF THESIS

Firstly, discussed here in Chapter 1 is an overview that summarizes the contents of this thesis. Each proceeding chapter is provided with a brief preface introducing the published work and how the publication contained in the chapter fit within the thesis theme.

Chapter 2 is presented as a published review article entitled "***NGF and proNGF: regulation of neuronal and neoplastic responses through receptor signaling***" *(Published in Advances in Biological Regulation)*. It serves as the background information of this thesis, reviewing the roles of proNGF/NGF and its receptors including TrkA, $p75^{NTR}$ and sortilin in modulating nervous system function and as active players in regulating neoplastic cell growth

Subsequently Chapter 3 is a study presented as published paper with the title ***"ProNGF correlates with Gleason score and is a potential driver of nerve infiltration in prostate cancer"*** *(Published in The American Journal of Pathology).* This study directly address one of the aims of this thesis that shows proNGF levels in prostate cancer is correlated with high Gleason score - a characteristic of aggressive prostate cancer. Also, high molecular weight forms of proNGF produced by prostate cancer cells are involved in neurotrophic-induced axonogenesis of neuronal cells in an *in vitro* co-culture assay. Interestingly, the correlation of proNGF levels with aggressive prostate cancer commensurate with the study demonstrated by Magnon and colleagues (2012). They showed higher density of nerve fibre infiltration in high Gleason scores of prostate cancers. Chapter 2 suggests that proNGF produced by prostate cancer cells may play a role in the infiltration of nerves observed in prostate cancer.

Provided by the evidence shown in Chapter 3, it was logical to investigate the phenomenon of nerve infiltration in other solid tumors such as breast cancer. Correspondingly, Chapter 4 shows that nerve fibres are an important component of the breast tumor microenvironment and that NGF actively plays a role in stimulating nerve fibre infiltration. This is presented in the published article entitled "***Nerve fibers Infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer"*** *(published in Molecular Oncology)*. It showed an association of nerve fibre infiltration with breast cancer aggressiveness - characterized by lymph node invasion or breast tumors with metastatic phenotype, in a cohort of clinically annotated invasive breast cancer. Interestingly, levels of NGF in breast tumors were associated with the presence of nerve fibres. Furthermore, produced and secreted NGF in breast cancer cells stimulated neuronal outgrowth *in vitro*.

Then the final discussion of this thesis is presented in Chapter 5 as a published work entitled "***Nerve-cancer cell crosstalk: a novel promoter of tumor progression***" *(Published in Cancer Research)* summarizing into perspective the findings elucidated in Chapter 3 and 4. In brief, the studies elucidated in this dissertation suggest that neurotrophic factors such as proNGF/NGF produced and secreted by tumors stimulate nerve fibres infiltration. While *vice versa*, infiltrating nerve fibers release neurotransmitters to induce tumor growth and dissemination.

CHAPTER 2 NGF AND PRONGF: REGULATION OF NEURONAL AND NEOPLASTIC RESPONSES THROUGH RECEPTOR SIGNALING

2.1 PREFACE

In this chapter is a published review entitled *"**NGF and proNGF: regulation of neuronal and neoplastic responses through receptor signaling**" (published in Advances in Biological Regulations)* serves as the relevant background to this thesis, which introduces the doctrines of nerve growth factor (NGF) and its precursor proNGF function. Described is the historical background of NGF as the pioneering member of the neurotrophin growth factor family. Discussed also are its roles in the regulation of neuronal death and survival mediated by its receptors tropomyosin receptor TrkA, pan-neurotrophin receptor $p75^{NTR}$, and the membrane protein sortilin. Whilst most studies about NGF and proNGF are focused on their role as regulators of neuronal functions, their involvement in the modulation of neoplastic activity is also reported under the sub-section "*NGF and breast cancer*" and "*ProNGF as an active growth factor*". The section entitled "*Neurotrophin-induced neurogenesis in tumor tissues*" begins with a tone asserting theoretical idea that suggests neurogenesis as a key player in the tumor microenvironment and proposes that proNGF/NGF are responsible for the tumor-induced neurogenesis. This hypothesis is tested in the proceeding chapters 3 and 4.

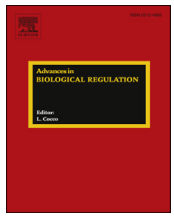
2.2 PUBLICATION

Advances in Biological Regulation 58 (2015) 16–27

Contents lists available at ScienceDirect

Advances in Biological Regulation

journal homepage: www.elsevier.com/locate/jbior



NGF and ProNGF: Regulation of neuronal and neoplastic responses through receptor signaling

Ralph A. Bradshaw [a, *], Jay Pundavela [b], Jordane Biarc [a, 1], Robert J. Chalkley [a], A.L. Burlingame [a], Hubert Hondermarck [b]

[a] Dept of Pharmaceutical Chemistry, UCSF, San Francisco, CA, USA
[b] School of Biomedical Sciences & Pharmacy, Hunter Medical Research Institute, Faculty of Health and Medicine, University of Newcastle, Australia

ARTICLE INFO

Article history:
Available online 20 November 2014

Keywords:
NGF
growth factor
receptor tyrosine kinase
signaling
phosphorylation

ABSTRACT

Nerve growth factor (NGF) and its precursor (proNGF) are primarily considered as regulators of neuronal function that induce their responses via the tyrosine kinase receptor TrkA and the pan-neurotrophin receptor p75NTR. It has been generally held that NGF exerts its effects primarily through TrkA, inducing a cascade of tyrosine kinase-initiated responses, while proNGF binds more strongly to p75NTR. When this latter entity interacts with a third receptor, sortilin, apoptotic responses are induced in contrast to the survival/differentiation associated with the other two. Recent studies have outlined portions of the downstream phosphoproteome of TrkA in the neuronal PC12 cells and have clarified the contribution of individual docking sites in the TrkA endodomain. The patterns observed showed a similarity with the profile induced by the epidermal growth factor receptor, which is extensively associated with oncogenesis. Indeed, as with other neurotrophic factors, the distribution of TrkA and p75NTR is not limited to neuronal tissue, thus providing an array of targets outside the nervous systems. One such source is breast cancer cells, in which NGF and proNGF stimulate breast cancer cell survival/growth and enhance cell invasion, respectively. This latter activity is exerted via TrkA (as opposed to p75NTR) in conjunction

Abbreviations: BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; RTK, receptor tyrosine kinase; SILAC, stable isotope labeling of amino acids in culture.

* Corresponding author. University of California San Francisco, 600 16th Street, N472 Genentech Hall, San Francisco, CA 94158-2517, USA. Tel.: +1 415 476 4813.

E-mail addresses: rab@cgl.ucsf.edu (R.A. Bradshaw), jay.pundavela@newcastle.edu.au (J. Pundavela), jordane.biarc@univ-lyon1.fr (J. Biarc), chalkley@cgl.ucsf.edu (R.J. Chalkley), alb@cgl.ucsf.edu (A.L. Burlingame), hubert.hondermarck@newcastle.edu.au (H. Hondermarck).

[1] Present address: UMR 5280, CNRS and Université de Lyon, 69622 Villeurbanne, France.

http://dx.doi.org/10.1016/j.jbior.2014.11.003

with sortilin. Another tissue overexpressing proNGF is prostate cancer and here the ability of cancer cells to induce neuritogenesis has been implicated in cancer progression. These studies show that the non-neuronal functions of proNGF/NGF are likely integrated with their neuronal activities and point to the clinical utility of these growth factors and their receptors as biomarkers and therapeutic targets for metastasis and cancer pain.

Introduction

Protein phosphorylation as a means to regulate and perpetrate cellular signaling mechanisms has been one of the dominant themes of biological research for several decades (Biarc et al., 2011). In humans alone, there are over 600 enzymes devoted to adding or removing this modification (approximately divided 5 to 1 between kinases and phosphatases) (Blume-Jensen and Hunter, 2001). These alterations can directly affect biological activity, as exemplified by the regulation of glycogen phosphorylase (which was the pioneering discovery that launched this field) (Krebs and Fischer, 1964), or, more often, exert their effects by altering protein–protein interactions. Moreover there has evolved an elaborate system, as manifested in specific recognition domains (Pawson, 2002), for recognizing key phosphorylation sites that lead to the formation of molecular complexes and that are required for the flux of information in dynamic signaling pathways. These domains, such as SH2 and PTB, are usually found in proteins that also have other domains that recognize different structural elements or contain effectors that generate new modifications or associations. The extent to which protein phosphorylations occur, even in resting (unstimulated) cells, in terms of both range and variety of sites, is sufficiently vast (thousands of loci) that it is unlikely that they are all of equal physiological significance (Gnad et al., 2011). Indeed many may be spurious, resulting from the substantial number of protein kinases that are active in any given cell at any given moment and the lack of tight substrate specificity for many of them. For the most part, these probably form a 'background' that may be of some general advantage to cells, since the accumulated negative charge from these sites may tend to keep cytoplasmic proteins away from membrane structures, which must be able to recruit, i.e. be available for binding of, certain signaling entities following stimulation in order to transmit their signals. Ascertaining which phosphorylations are essential and which are not (and which are introduced by which kinase) remains a singularly important challenge.

Protein phosphorylations in mammalian species occur primarily on serine, threonine and tyrosine residues and in that relative order of abundance (serine phosphorylations being the most prevalent). Protein kinases with specificity for tyrosine make up about 20% of the family and are found both as cytoplasmic and integral membrane bound entities. This latter group constitute the so-called receptor tyrosine kinases (RTKs), which are subdivided into 20 families; some of these contain only a single member, such as MUSK or RET while the ephrins have more than a dozen members. Likewise the ligand families that activate these entities also can be singular in nature or spread among multiple homologous members.

Although the overall organization of the RTKs is generally the same, with each containing an extracellular (or exo-) domain, a transmembrane domain and an intracellular (or endo-) domain, there are notable distinguishing differences in the intra-domain organization of the exo- and endo-moieties. The exodomains, whose function is basically to provide the recognition and subsequent binding of the activating ligands, are composed of many different folding motifs (sometimes in tandem arrays) and these show considerable variability, although domains of the same basic motif are found in different families. On the other hand, the endodomains, which all contain the eponymous tyrosine kinase, actually share little similarity in the non-kinase regions that are found between the transmembrane and kinase domains (juxtamembrane domain) and the kinase domain and the C-terminus. These

segments vary considerably in length and in function. Moreover, the distribution of tyrosine residues, a subset of which are phosphorylated in each case and generally provide docking sites for adaptor/scaffold/effector moieties, are also significantly different. This provides, in turn, a number of distinct means for propagating the signal from that receptor (Bradshaw et al., 2013). In the light of this diversity, it is somewhat surprising that there is considerable uniformity in the downstream pathways that are activated by different RTK families. In the main, RTKs stimulate three main pathways: the activation of ERKs via Ras, GTP binding proteins and several other kinases; the activation of phospholipase Cγ with the resulting production of diacylglycerol and inositol triphosphate from the cleavage of phosphoinositides; and the activation of the several Akt pathways via the agency of phosphoinositide-3-kinase (PI3K) (Schlessinger, 2000; Choudhary and Mann, 2010). These events are accompanied by a broad stimulation of protein kinases, producing extensive modifications (primarily on serine and threonine residues) as well as other reversible modifications, such as N^{ε}-acetylation, ubiquitination and O-glycosylation with GlcNAc (Zeidan & Hart, 2010). The extent of these alterations and the full description of their impact on cellular activities and responses in any system remain to be elucidated.

Neurotrophins and their receptors

Neurotrophic factors are a broad group of growth factors and cytokines whose principal targets are neurons of the peripheral and central nervous systems. They can stimulate neurite growth, maintain viability and induce differentiation, among other activities. The first such substance to be defined, and the prototype of the class, was NGF (Levi-Montalcini, 1987). It was originally observed in two mouse tumor cell lines by its ability to induce fiber outgrowth of sympathetic and sensory neurons, but its discovery in the male mouse submandibular gland opened the way for the detailed molecular characterization (Shooter, 2001), including sequence analysis (Angeletti and Bradshaw, 1971), of the mature protein. Cloning experiments established that it was, not surprisingly, elaborated as a prepro protein, with a signal sequence of 19 residues and a pro segment of 120 residues (Scott et al., 1983; Ullrich et al., 1983). Several years later, a homolog of NGF that was primarily found in the brain, was isolated, characterized and designated brain-derived neurotrophic factor (BDNF) (Barde et al., 1982). Molecular cloning experiments defined two more members of this family, neurotrophins 3 and 4 (NT3 and -4) (Maisonpierre et al., 1991; Ip et al., 1992). Although early observations suggested that NGF behaved like a hormone (Frazier et al., 1972) and iodinated tracer-binding experiments supported the presence of a cell surface receptor, its identification and characterization proceeded relatively slowly. This was due in part to a variety of measurements that yielded conflicting results with regard to both binding properties and molecular mass (Raffioni et al., 1993). On the one hand, there was compelling evidence that biological activity (generally defined as neurite outgrowth from PC12 cells) was associated with a molecule of about ~130 kDa (Kouchalakos and Bradshaw, 1986); on the other, there was strong evidence for the existence of a receptor protein of half that mass and this was basically confirmed by cloning experiments (Chao et al., 1986; Radeke et al., 1987). This latter entity eventually became known as p75NTR or the pan-neurotrophin receptor because it bound all four members of the neurotrophin family with about the same affinity. However, it did not contain a kinase or other known effectors as part of its endodomain. The enigma was finally resolved in 1991 when TrkA, an RTK (of the molecular mass previously predicted) was identified and cloned (Kaplan et al., 1991; Klein et al., 1991). Two additional members of this RTK family, TrkB and C with specificities for the other neurotrophins were eventually identified. The final participant in this group is sortilin (also known as NTSR3 for neurotensin receptor 3 or GP110 for glycoprotein 110). It has multiple functions and binds several different types of ligands including proNGF and proBDNF. This interaction is involved in apoptosis and appears to function in concert with p75NTR (Hempstead, 2014). In this regard, it has been shown that the pan-neurotrophin receptor also binds more avidly to the proneurotrophins than it does to the corresponding mature forms. A portrait of this group of ligands and receptors is shown in Fig 1.

The neurotrophins are expressed in a broad array of tissues, consistent with the view that they mainly function as target-derived survival factors (Kaplan and Miller, 2000; Reichardt, 2006; Hempstead, 2014). NGF was initially envisioned as a peripheral nervous system agent but it is clear that it has some central nervous system functions as well. In contrast, BDNF is primarily important in the brain, and as such has received considerable attention as a target for common CNS maladies, such

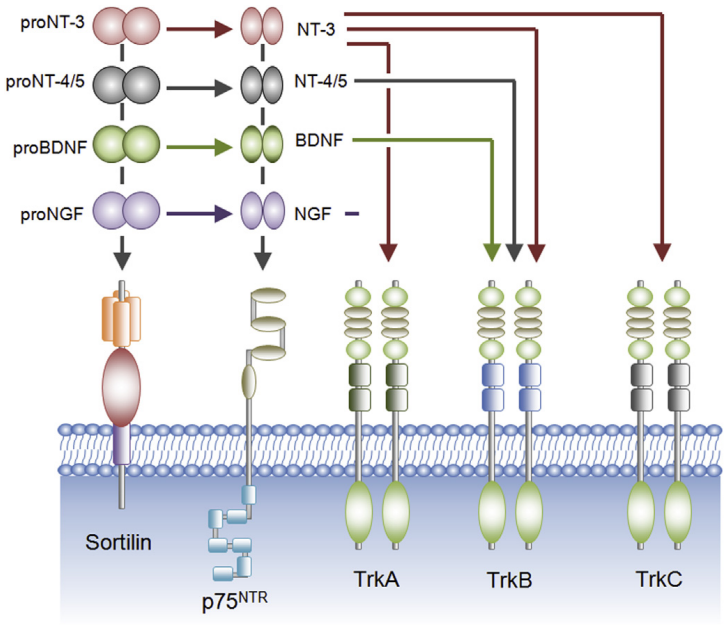


Fig. 1. Binding of neurotrophins and proneurotrophins to Trk receptors and p75NTR. NGF, BDNF, NT-3, NT-4/5 as well as their respective precursors (proNGF, proBDNF, proNT, proNT-4/5) all bind to the pan-neurotrophin receptor $p75^{NTR}$ while Trk receptors bind neurotrophins with different specificities. Sortilin binds only the precursor forms.

as Parkinson's disease. As a result, its receptors are commonly found on responsive neurons, although they are also found on other non-neuronal tissues too. The potential importance of NGF (and proNGF) in the responses of both normal and neoplastic non-neuronal tissues will be elaborated on in subsequent sections below.

TrkA induced signaling

As with other RTKs, the activation of TrkA by ligand binding results in the formation of a number of phosphorylated tyrosines on its endodomain, most notably the three found in the activation loop and those at position 490 in the juxtamembrane domain and 785 in the C-terminal domain. It is generally held that these result from autocatalysis but the involvement of another tyrosine kinase (activated by the receptor kinase) has not been ruled out. Although these modifications also lead to other tyrosine phosphorylations, a much more striking outcome is the plethora of downstream phosphorylations that occur on a host of intracellular proteins. Of course there is a high level of 'baseline' (unstimulated) modifications and those that might relate to growth factor stimulated responses should be reflected in significant change, i.e. be either up- or down-regulated, from the control. In situations of acute stimulation (stimulus added as a signal bolus addition) the total amount of phosphorylation peaks about 20 min after addition of the ligand. In order to better define the TrkA phosphoproteome at this time point, PC12 cells, a well-studied paradigm with many neuronal characteristics (including their response to NGF) were engineered to express hybrid TrkA receptors that were designed to specifically avoid endogenous signaling and to allow dissection of the participating tyrosines. Basically, the extracellular domain of the human platelet-derived growth factor (PDGF) receptor was fused to the transmembrane and intracellular domain of rat TrkA (termed PTR) and stably transfected into PC12 cells. Derivatives in which Y490 and Y490/Y785 were mutated to phenylalanine were also constructed and expressed. The chimeric receptors were appropriately responsive to PDGF (but not untransfected cells which have no PDGF receptors) in all cases. The phosphoproteome changes induced, compared to unstimulated cells, were quantified by growing each transfected cell line in media with isotopically labeled amino acids (SILAC) and measuring the released tryptic peptides by MS/MS following TiO_2 enrichment (Biarc et al., 2012). As shown in Fig 2, there were 988 peptides with greater than a 2-fold

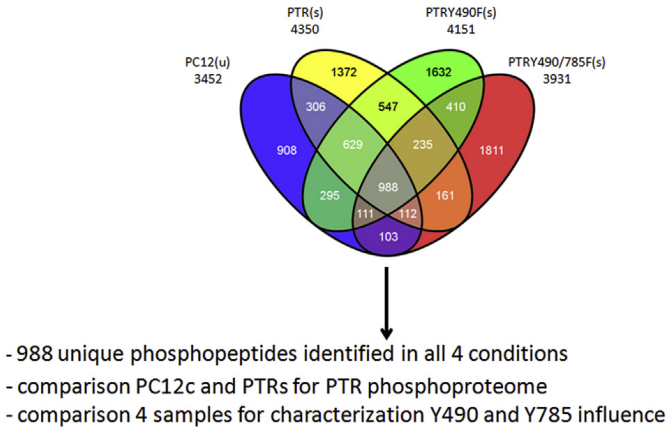


Fig. 2. Overlap of phosphopeptides identified upon receptor stimulation. Venn diagram describing the phosphopeptides identified in PC12 cells (PC12c), PC12 cells stably transfected with chimeric receptor PTR stimulated for 20 min with PDGF-BB (PTRs), PTR Y490F stimulated (PTR Y490F(s)) or PTR Y490F/Y785F stimulated with a peptide false-positive rate of 0.5%. 988 phosphopeptides were identified in all four conditions. Adapted from Biarc et al. (2013).

change that were identified in all four samples (unstimulated PC12 cells and stimulated samples of the wild type receptor and the two mutants). Further analyses of these samples underscored the central role of Y490 in activating the Erks and effecting changes in transcription while Y785 (which is known to activate PLCγ) is more involved in cell cycle/mitotic control. Interestingly these studies also established that there was still signaling by the double mutant, indicating at least one additional docking site (perhaps involving the activation loop tyrosines) that was strongly manifested in CK2 regulation (Biarc et al., 2013).

One analysis that was particularly interesting was a comparison of these findings with a similar set of phosphopeptide identifications from the stimulation of HeLa cells by EGF at the same time point (Olsen et al., 2006). Plotted using the catalytic specificity motifs of 16 groups of kinases, the data revealed a high degree of similarity, suggesting that the kinases stimulated were substantially overlapping, despite the fact that the two cell types were different and from different species (rat vs. human) (Fig 3). In view of the heavy involvement of EGF in many cancers, as well as some other RTK members (Drake et al., 2014), it raises questions of whether there might not be a similar involvement of NGF and the other neurotrophins in cancer as well. Obviously, since their normal targets (neurons) are basically post-mitotic, they would not, at first pass, appear to be good candidates for such a role. However, this would ignore the fact that there are well established roles for NGF outside both the central and peripheral nervous systems and this provides potential opportunities for both ligands and receptors to be of oncologic significance (Kruttgen et al., 2006). Indeed, essentially all of the so-called neurotrophic factors appear to function with this kind of dual functionality, which may be an important consideration in their deployment as potential biomarkers or therapeutic targets.

NGF and breast cancer

The first indication of NGF involvement in breast cancer was the discovery of a stimulatory effect on the proliferation of several mammary tumor-derived epithelial cell lines (Descamps et al., 1998). These cells expressed both TrkA and the p75NTR receptor and the effects were clearly demonstrated to require the activation of the MAP kinases via the TrkA receptor. Subsequently, it was shown that the activation of p75NTR (and the transcription factor NF-κB) lead to an anti-apoptotic effect that was dependent on TRADD (Descamps et al., 2001; El Yazidi-Belkoura et al., 2003). Thus in breast cancer cells, this dual activation of TrkA and p75 leads to the stimulation of cell proliferation and survival, respectively, a situation in which the two receptors initiate separate signaling pathways that ultimately lead to different biological effects, albeit that there are interconnections between them. The direct demonstration that breast cancer cells produce NGF thus provides all the elements of an autocrine loop

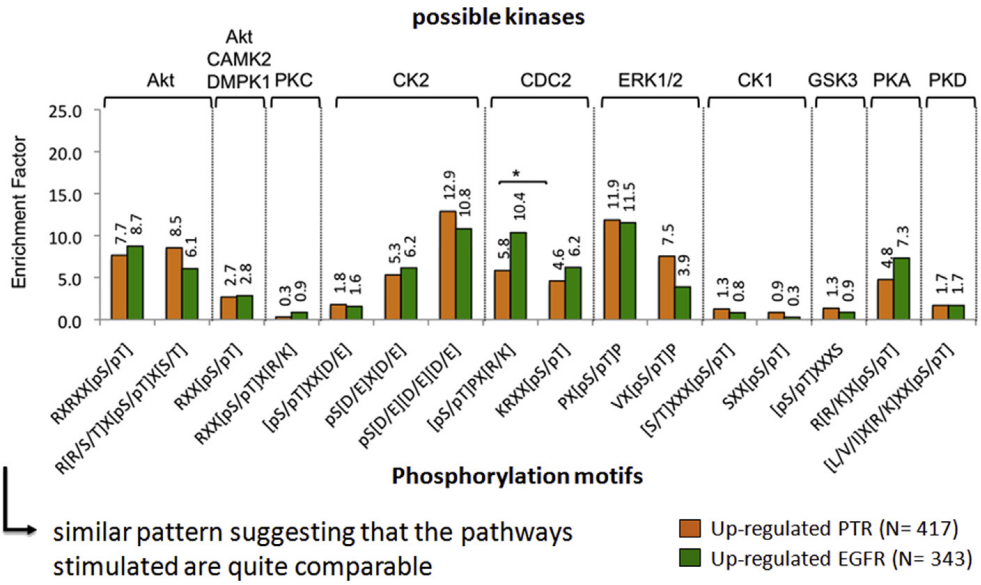


Fig. 3. Phosphorylation motifs. Sixteen phosphorylation motifs modified by different kinases that are represented at the top of the figure were analyzed for the regulated phosphopeptides by determining their enrichment in each population. Plotted is the enrichment factor (how frequently phosphorylation in a particular motif was observed in comparison to the motif's frequency in all rat proteins) in up-regulated phosphopeptides upon stimulation of the TrkA chimera in PC12 cells (orange bars) and EGFR in Hela cells (Olsen et al., 2006) (green bars). Adapted from Biarc et al. (2013). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

involving NGF and its receptors (Dolle et al., 2003) and as its inhibition results in a diminished tumor growth in a preclinical animal model (Adriaenssens et al., 2008), it underscores the potential value of NGF as a therapeutic target.

The role of TrkA in the signaling processes of breast cancer cells appears to be in part different than in their neuronal counterparts. Com et al. (Com et al., 2007) used proteomics to determine a number of TrkA signaling partners in MCF-7 breast cancer cells, in particular Ku70; a protein involved in DNA repair that has also been found to be associated with EGF receptor signaling (Bandyopadhyay et al., 1998). Interestingly it is not involved in TrkA signaling in PC12 cells but clearly plays a role in the prevention of breast cancer cell apoptosis. Indeed, in the absence of this regulator, TrkA can act as a pro-apoptotic agent. Therefore it is not only p75NTR, but also TrkA that can participate in the resistance to apoptosis induced by NGF. In a separate study, Lagadec et al. (Lagadec et al., 2010) identified a second DNA repair protein, Ku86, in tumor cells over expressing TrkA and showed that PI3K-Akt and ERK/p38 MAP kinases were activated and required for the maintenance of a more aggressive cellular phenotype. In addition to the stimulating effect of NGF on breast cancer cell survival and proliferation, altered expression of TrkA is also associated with tumor progression to effusion and clearly enhances growth and metastasis of breast cancer cells (Davidson et al., 2004; Lagadec et al., 2010).

In neuronal cells, there is considerable debate about the role of p75NTR as a regulator of TrkA activity and whether there is a direct interaction between the two receptors (Reichardt, 2006). While they certainly exert an effect on each other with respect to function and response, there is no compelling basis to assume that physical complexes actually form. This is also true in breast cancer cells but there siRNA or pharmacological inhibitors have also established that there is no particular effect of one receptor on the functionality of the other receptor. Therefore, it appears that in breast cancer cells, TrkA and p75 are working rather independently from one another (Fig. 4B).

ProNGF as an active growth factor

Although it is not surprising that the neurotrophins are synthesized as precursors that contain pro-domains in addition to their mature sequences, it is unusual that these entities are important ligands in

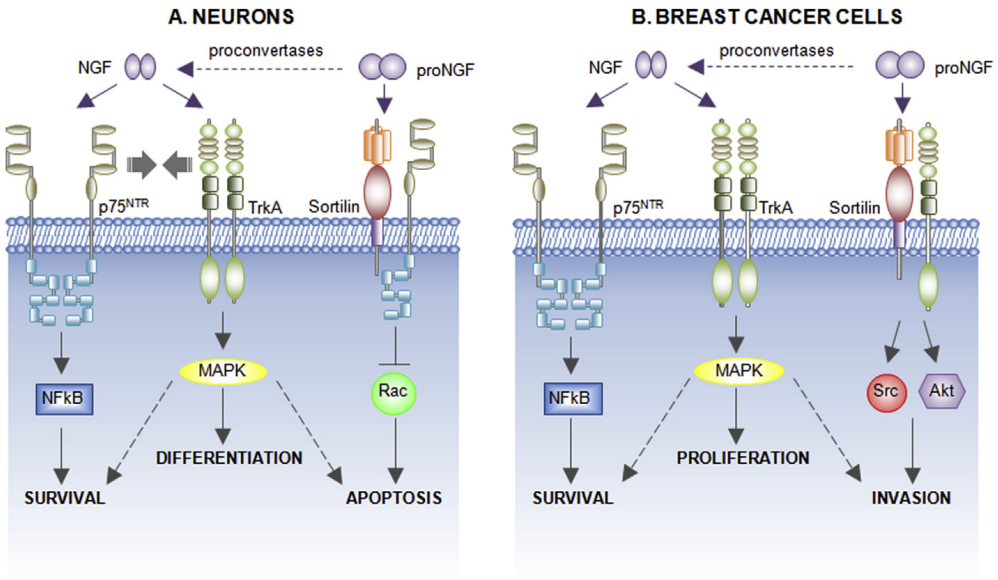


Fig. 4. ProNGF/NGF signaling in neurons and breast cancer cells. A) In neurons NGF stimulates survival and differentiation through TrkA and p75NTR and via a signaling involving the MAP kinases and NFkB. ProNGF stimulate a complex between p75NTR and sortilin that leads to the inhibition of RAC (Rho GTPase). B) In breast cancer cells, NGF stimulates TrkA and p75NTR leading to the activation of cell proliferation and survival, respectively. ProNGF binds to a complex TrkA/sortilin to stimulate cancer cell migration and invasion via the activation of Src and Akt.

their own right. A precursor of NGF was first detected in 1977 by immunoprecipitation of radiolabeled protein synthesized in tissue samples of mouse submaxillary gland (Berger and Shooter, 1977) and was subsequently confirmed by cloning experiments, which provided molecular details (Scott et al., 1983; Ullrich et al., 1983). It has since been detected in a number of tissues (Hempstead, 2014) and was reported to be the sole detectable form (by Western blot) of the protein in the brain (Fahnestock et al., 2001). Because there are two alternative spliced forms along with various glycosylated intermediates, proNGF can be observed in multiple forms. The demonstration that proNGF had a higher affinity for the p75NTR receptor than TrkA, and further is bound to sortilin via its pro peptide to promote pro-apoptotic activities in concert with p75NTR, provided a clear rationale for its prevalence. It is still unclear what regulates the processing events (or lack thereof), which are thought to be performed by furins and proconvertases intracellularly (Seidah et al., 1996) and by plasmin and MMPs after secretion (Teng et al., 2010) and determine the amounts of proNGF vs. the mature form in any given situation. The end result in neuronal cells is that proNGF, in the absence of processing, is an active product that promotes apoptosis via p75NTR/sortilin complexes and counters the effect of NGF, acting via TrkA or p75NTR, to stimulate survival and differentiation (Fig 4A).

While most attention has been focused on the role of proneurotrophins in the nervous system, proNGF has been associated with other types of tissues as well. Both dermal and cardiac responses have been described (Hempstead, 2014). However, a more compelling involvement in the behavior of several tumor types suggests that, like several other members of the greater RTK family including both ligands and receptors, it may be of much more significance in the management of oncological pathologies (Kruttgen et al., 2006). For example, proNGF can stimulate invasion of melanoma cells through a mechanism involving p75NTR and sortilin (Truzzi et al., 2008). These cells are also of neuroectodermal origin and express all the members of the neurotrophin family and its three distinct receptors and utilize both TrkA and p75NTR in promoting proliferation. In this case the p75NTR-sortilin complex is implicated in promoting migration.

As described above, breast cancers express and respond to NGF, and therefore the discovery that proNGF is secreted by tumor cells was not overly surprising (Demont et al., 2012). However the determination that it stimulates their migration/invasion through an autocrine loop mediated by TrkA

and sortilin was unexpected. This somewhat controversial observation is the first indication of a biologically significant TrkA-sortilin partnership. The signaling pathway requires the phosphorylation of TrkA as well as the activation of Src and Akt, but not the MAP-kinases. Moreover, in contrast to melanoma cells, p75NTR is not involved. In addition, a comparison between proNGF levels and clinicopathological parameters revealed a correlation with lymph node invasion. In invasive ductal carcinomas, which represent the majority of breast cancers, there was no correlation with histological grade, tumor value, axillary lymph node status, age and presence of estrogen receptors, although a statistically significant association was obtained between the quantity of proNGF and lymph node invasion, suggesting a link to metastasis (Demont et al., 2012). Indeed, proNGF may serve as a biomarker of metastasis and possibly as a therapeutic target in breast cancer. As described below, prostate tumors also express proNGF.

Neurotrophin-induced neurogenesis in tumor tissues

The tumor microenvironment represents an additional area of great importance in understanding the factors controlling neoplastic tissue growth and progression, particularly as they relate to metastases and all factors and elements involved in these processes impact it (Swartz et al., 2012). In breast cancer, tumor neovascularization and macrophage invasion are generally held to be the most important elements of the microenvironment influencing tumor development, and NGF/proNGF contribute to both of these (Hondermarck, 2012). Angiogenesis requires the activation and proliferation of endothelial cells (usually recruited from the pre-existing vascular bed) and vascular endothelial cell growth factor (VEGF) and the fibroblast growth factors (FGFs), that stimulate other members of the RTK family, are key components of this activity. Indeed, inhibiting angiogenesis has been an important target for cancer therapeutics (Gimbrone et al., 1972). NGF has also been reported to promote angiogenesis and/or induce the expression of proangiogenic molecules in several tissues (Cantarella et al., 2002), including breast cancer (Romon et al., 2010). Related to tumor angiogenesis is the facilitation of the infiltration of immune cells. The link between inflammation and cancer involves a variety of cytokines and chemokines and NGF is produced by various immune cells (Leon et al., 1994; Nilsson et al., 1997). It has recently been shown that breast cancer NGF can stimulate TrkA signaling in tumor-associated macrophages, increasing IL-10 production (Ley et al., 2013).

A third potential contributor to the tumor microenvironment is from nerve fibers induced to infiltrate the tumor. The reverse situation, perineural invasion, whereby tumors infiltrate and follow nerve fibers occurs frequently and has been well documented in pancreatic, prostatic and breast cancer (Villers et al., 1989; Karak et al., 2010). The occurrence of perineural invasion does not generally lead to a good prognosis. Nerve fibers are commonly found in the microenvironment, but there is a paucity of information about what they might contribute to the growth and expansion of tumors. Ayala et al. (Ayala et al., 2008; Magnon et al., 2013) were among the first to suggest tumors promote neurogenesis in prostate cancer and suggested the overexpression of semaphorin 4F might be mechanistically responsible. Recently, Magnon et al. (Magnon et al., 2013) reported a study of autonomic nerve formation in prostate cancer, establishing that fibers from both the sympathetic (adrenergic) and parasympathetic (cholinergic) systems were present, with the former dominating the early stages. The density of these fibers was directly correlated to the Gleason prostate cancer score, and in an animal model, denervation resulted in a decrease in tumor engraftment and metastasis. Thus, these new autonomic nerve projections affected both cancer initiation and progression.

The mechanisms responsible for stimulating the growth of these peripheral neurons into the prostate tumors were not addressed. Entschladen et al. (Entschladen et al., 2006) put forth the idea that neurogenesis (they termed it neoneurogenesis) could be induced by tumors through the production of neurotrophic factors. It had already been reported (Delsite and Djakiew, 1999) that a proNGF molecule of 22 kDa is expressed by human prostatic stromal cells, as detected immunologically, but mature NGF, which would be expected to be the agent capable of attracting sympathetic and/or sensory neurites was not detected in these studies. To address whether NGF or proNGF may be involved in prostate tumor-directed neurogenesis, a cohort of 120 human prostate samples was examined by immunohistochemistry (Pundavela et al., 2014). ProNGF was readily detected in the cytoplasm of the cancer cells but much less so in the stromal cells. Importantly quantification of these observations indicated

that the levels detected correlated with the Gleason scores of these samples ($n = 104$, coefficient of correlation $\tau B = 0.51$) and this pattern matched the neurite invasion data of Magnon et al. (Magnon et al., 2013). In keeping with previous observations (Delsite and Djakiew, 1999), mature NGF was not detected in prostate cancer cells. Western blot analysis of three prostate cancer-derived cell lines compared with normal prostate epithelial cells, transformed non-tumorigenic prostate epithelial cells and benign prostate hyperplasia (BPH) cells indicated that the tumor and BPH cells showed a prominent band at 60 kDa that was largely absent in the normal cells. This form of proNGF was previously described in uterine samples (Lobos et al., 2005); indeed several high molecular mass forms have been observed that are, at least in part, derived from glycosylation and alternative splicing. However, the detailed molecular characterization of the proNGF produced by prostate tumor cells has not been determined and it could contain other modifications as well. Determining the nature of the alterations that lead to the higher molecular mass forms will be an important step in evaluating the usefulness of proNGF as prostate cancer biomarker.

To ascertain whether the proNGF identified immunologically was capable of inducing the peripheral neuron infiltration of the tumors (Magnon et al., 2013), the prostate cancer cell line PC-3 was incubated with two NGF-responsive cell lines, PC-12 and 50B11, in Transwell Boyden chambers (Pundavela et al., 2014). Both of these paradigms extend neurites when exposed to germane neurotrophic agents, such as NGF. PC-3 cells were able to induce neurite outgrowth with both test cell lines whereas control normal cells did not. Moreover the responses were inhibited by anti-proNGF sera but were not affected by an isotype anti-sera. Clearly the proNGF observed to be present in prostate tumor cells is exported in a manner sufficient to induce the nerve infiltration observed (Magnon et al., 2013). However, it is not known if proNGF is acting on its own to stimulate neurite outgrowth in prostate tumors or if it requires processing to mature NGF.

Given the responses to prostate tumors, it is reasonable to assume that other tumors might also induce neurogenesis from peripheral neurons that could impact tumor growth and progression. Albo et al. (Albo et al., 2011) and Tomita et al. (Tomita, 2012) have reported neoneurogenesis in colon cancer and Zhao et al. (Zhao et al., 2014) have very recently made similar observations for breast cancer where they observed PGP 9.5 positive fibers in over 60% of a cohort of 144 cases of invasive ductal carcinoma. In an independent study,[2] nerve fibers were imaged in a cohort of primary invasive breast cancers by immunohistochemistry with the same neuronal marker. Neurites were detected in 20% of tumors and there was an association with NGF expression and lymph node invasion, suggesting a relationship with the metastatic potential. Although broader studies will be required to confirm and extend these observations, it already seems clear that many types of tumors have the potential to express NGF (and/or proNGF) and that these factors, in turn, may induce peripheral nerve infiltration into the tumor microenvironment, resulting in further stimulation of tumor growth and metastases. Such effects may not be limited to the neurotrophins but may be stimulated by other neurotrophic factors as well.

ProNGF/NGF stimulated nerve infiltration in solid tumors may also participate in cancer pain. Indeed NGF is also a mediator of pain that acts though the activation of TrkA in endings of sensory neurons (Pezet & McMahon, 2006). Blocking antibodies against NGF, and pharmacological inhibitors against TrkA, have been developed and some are already in clinical trials for their potent analgesic effect in rheumatoid and back pain (Longo and Massa, 2013). Interestingly, in the mouse it has been shown that anti-NGF antibodies can decrease the pain caused by bone metastasis and to attenuate bone destruction (Jimenez-Andrade et al., 2011; McCaffrey et al., 2014). Therefore targeting NGF/proNGF in cancer could also have an additional impact by reducing cancer pain.

Conclusions

NGF and its precursor, proNGF, clearly have multiple roles in both neuronal and non-neuronal targets as exerted through three receptor types. Importantly these seem to manifest themselves in different ways and with different phenotypic responses. These differences are most acute when

[2] Pundavela, J., Roselli, S., Faulkner, S., Attia, J., Scott, R. J., Forbes, J. F., Bradshaw, R. A., Walker, M. M., Jobling, P. and Hondermarck, H., submitted for publication.

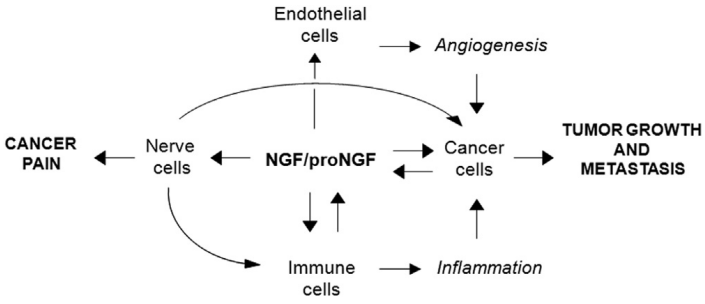


Fig. 5. ProNGF/NGF impact on cancer progression. ProNGF/NGF produced by cancer cells activates cancer cells growth and dissemination via an autocrine loop of stimulation, and stimulate various cell types in the tumor microenvironment. Immune cells, endothelial cells and nerves in the tumor microenvironment are activated, leading to the stimulation of inflammation, neoangiogenesis and nerve infiltration. The presence of nerve fibers in the tumor microenvironment could contribute to the feeling of pain in and around the tumor.

comparing normal and neoplastic tissues. Thus, as shown in Fig 5, NGF and proNGF (and presumably other neurotrophic factors) can directly affect tumor cells or they can influence the composition and responses of the cells that are an important part of the microenvironment, stimulating such tumor sensitive processes as angiogenesis, immune responses and pain. This places NGF/proNGF in a central role for the diagnosis and management of many breast and prostate cancers and its detection and inhibition may become important clinically.

Acknowledgments

This work was supported in part by the University of Newcastle Australia, the Hunter Cancer Research Alliance, the Biomedical Technology Research Centers Program of the USPHS National Institute of General Medical Sciences, 8P41GM103481, and NIH 1S10OD016229.

References

Adriaenssens E, Vanhecke E, Saule P, Mougel A, Page A, Romon R, et al. Nerve growth factor is a potential therapeutic target in breast cancer. Cancer Res 2008;68(2):346–51.
Albo D, Akay CL, Marshall CL, Wilks JA, Verstovsek G, Liu H, et al. Neurogenesis in colorectal cancer is a marker of aggressive tumor behavior and poor outcomes. Cancer 2011;117(21):4834–45.
Angeletti RH, Bradshaw RA. Nerve growth factor from mouse submaxillary gland: amino acid sequence. Proc Natl Acad Sci U S A 1971;68(10):2417–20.
Ayala GE, Dai H, Powell M, Li R, Ding Y, Wheeler TM, et al. Cancer-related axonogenesis and neurogenesis in prostate cancer. Clin Cancer Res 2008;14(23):7593–603.
Bandyopadhyay D, Mandal M, Adam L, Mendelsohn J, Kumar R. Physical interaction between epidermal growth factor receptor and DNA-dependent protein kinase in mammalian cells. J Biol Chem 1998;273(3):1568–73.
Barde YA, Edgar D, Thoenen H. Purification of a new neurotrophic factor from mammalian brain. Embo J 1982;1(5):549–53.
Berger EA, Shooter EM. Evidence for pro-beta-nerve growth factor, a biosynthetic precursor to beta-nerve growth factor. Proc Natl Acad Sci U S A 1977;74(9):3647–51.
Biarc J, Chalkley RJ, Burlingame AL, Bradshaw RA. Receptor tyrosine kinase signaling - a proteomic perspective. Adv Enzyme Regul 2011;51:293–305.
Biarc J, Chalkley RJ, Burlingame AL, Bradshaw RA. The induction of Serine/Threonine protein phosphorylations by a PDGFR/TrkA chimera in stably transfected PC12 cells. Mol Cell Proteomics 2012;11:15–30.
Biarc J, Chalkley RJ, Burlingame AL, Bradshaw RA. Dissecting the roles of tyrosines 490 and 785 of TrkA protein in the induction of downstream protein phosphorylation using chimeric receptors. J Biol Chem 2013;288(23):16606–18.
Blume-Jensen p, Hunter T. Oncogenic kinase signaling. Nature 2001;411:355–65.
Bradshaw RA, Chalkley RJ, Biarc J, Burlingame AL. Receptor tyrosine kinase signaling mechanisms: devolving TrkA responses with phosphoproteomics. Adv Biol Regul 2013;53(1):87–96.
Cantarella G, Lempereur L, Presta M, Ribatti D, Lombardo G, Lazarovici P, et al. Nerve growth factor-endothelial cell interaction leads to angiogenesis in vitro and in vivo. Faseb J 2002;16(10):1307–9.
Chao MV, Bothwell MA, Ross AH, Koprowski H, Lanahan AA, Buck CR, et al. Gene transfer and molecular cloning of the human NGF receptor. Science 1986;232(4749):518–21.
Choudhary C, Mann M. Decoding signalling networks by mass spectrometry-based proteomics. Nat Rev Mol Cell Biol 2010;11:427–39.

Com E, Lagadec C, Page A, El Yazidi-Belkoura I, Slomianny C, Spencer A, et al. Nerve growth factor receptor TrkA signaling in breast cancer cells involves Ku70 to prevent apoptosis. Mol Cell Proteomics 2007;6(11):1842–54.
Davidson B, Reich R, Lazarovici P, Ann Florenes V, Nielsen S, Nesland JM. Altered expression and activation of the nerve growth factor receptors TrkA and p75 provide the first evidence of tumor progression to effusion in breast carcinoma. Breast Cancer Res Treat 2004;83(2):119–28.
Delsite R, Djakiew D. Characterization of nerve growth factor precursor protein expression by human prostate stromal cells: a role in selective neurotrophin stimulation of prostate epithelial cell growth. Prostate 1999;41(1):39–48.
Demont Y, Corbet C, Page A, Ataman-Onal Y, Choquet-Kastylevsky G, Fliniaux I, et al. Pro-nerve growth factor induces autocrine stimulation of breast cancer cell invasion through tropomyosin-related kinase A (TrkA) and sortilin protein. J Biol Chem 2012;287(3):1923–31.
Descamps S, Lebourhis X, Delehedde M, Boilly B, Hondermarck H. Nerve growth factor is mitogenic for cancerous but not normal human breast epithelial cells. J Biol Chem 1998;273(27):16659–62.
Descamps S, Toillon RA, Adriaenssens E, Pawlowski V, Cool SM, Nurcombe V, et al. Nerve growth factor stimulates proliferation and survival of human breast cancer cells through two distinct signaling pathways. J Biol Chem 2001;276(21):17864–70.
Dolle L, El Yazidi-Belkoura I, Adriaenssens E, Nurcombe V, Hondermarck H. Nerve growth factor overexpression and autocrine loop in breast cancer cells. Oncogene 2003;22(36):5592–601.
Drake JM, Lee JK, Witte ON. Clinical targeting of mutated and wild-type protein tyrosine kinases in cancer. Mol Cell Biol 2014; 34(10):1722–32.
El Yazidi-Belkoura I, Adriaenssens E, Dolle L, Descamps S, Hondermarck H. Tumor necrosis factor receptor-associated death domain protein is involved in the neurotrophin receptor-mediated antiapoptotic activity of nerve growth factor in breast cancer cells. J Biol Chem 2003;278(19):16952–6.
Entschladen F, Palm D, Lang K, Drell TLt, Zaenker KS. Neoneurogenesis: tumors may initiate their own innervation by the release of neurotrophic factors in analogy to lymphangiogenesis and neoangiogenesis. Med Hypotheses 2006;67(1):33–5.
Fahnestock M, Michalski B, Xu B, Coughlin MD. The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer's disease. Mol Cell Neurosci 2001;18(2):210–20.
Frazier WA, Angeletti RH, Bradshaw RA. Nerve growth factor and insulin. Science 1972;176(4034):482–8.
Gimbrone Jr MA, Leapman SB, Cotran RS, Folkman J. Tumor dormancy in vivo by prevention of neovascularization. J Exp Med 1972;136(2):261–76.
Gnad F, Gunawardena J, Mann M. PHOSIDA 2011: the posttranslational modification database. Nucleic Acids Res 2011; 39(Database issue):D253–60.
Hempstead BL. Deciphering proneurotrophin actions. Handb Exp Pharmacol 2014;220:17–32.
Hondermarck H. Neurotrophins and their receptors in breast cancer. Cytokine Growth Factor Rev 2012;23(6):357–65.
Ip NY, Ibanez CF, Nye SH, McClain J, Jones PF, Gies DR, et al. Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. Proc Natl Acad Sci U S A 1992;89(7):3060–4.
Jimenez-Andrade JM, Ghilardi JR, Castaneda-Corral G, Kuskowski MA, Mantyh PW. Preventive or late administration of anti-NGF therapy attenuates tumor-induced nerve sprouting, neuroma formation, and cancer pain. Pain 2011;152(11):2564–74.
Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF. The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science 1991;252(5005):554–8.
Kaplan DR, Miller FD. Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 2000;10(3):381–91.
Karak SG, Quatrano N, Buckley J, Ricci Jr A. Prevalence and significance of perineural invasion in invasive breast carcinoma. Conn Med 2010;74(1):17–21.
Klein R, Jing SQ, Nanduri V, O'Rourke E, Barbacid M. The trk proto-oncogene encodes a receptor for nerve growth factor. Cell 1991;65(1):189–97.
Kouchalakos RN, Bradshaw RA. Nerve growth factor receptor from rabbit sympathetic ganglia membranes. Relationship between subforms. J Biol Chem 1986;261(34):16054–9.
Krebs EG, Fischer EH. Phosphorylase and related enzymes of glycogen metabolism. Vitam Horm 1964;22:399–410.
Kruttgen A, Schneider I, Weis J. The dark side of the NGF family: neurotrophins in neoplasias. Brain Pathol 2006;16(4):304–10.
Lagadec C, Romon R, Tastet C, Meignan S, Com E, Page A, et al. Ku86 is important for TrkA overexpression-induced breast cancer cell invasion. Proteomics Clin Appl 2010;4(6–7):580–90.
Leon A, Buriani A, Dal Toso R, Fabris M, Romanello S, Aloe L, et al. Mast cells synthesize, store, and release nerve growth factor. Proc Natl Acad Sci U S A 1994;91(9):3739–43.
Levi-Montalcini R. The nerve growth factor 35 years later. Science 1987;237(4819):1154–62.
Ley S, Weigert A, Weichand B, Henke N, Mille-Baker B, Janssen RA, et al. The role of TRKA signaling in IL-10 production by apoptotic tumor cell-activated macrophages. Oncogene 2013;32(5):631–40.
Lobos E, Gebhardt C, Kluge A, Spanel-Borowski K. Expression of nerve growth factor (NGF) isoforms in the rat uterus during pregnancy: accumulation of precursor proNGF. Endocrinology 2005;146(4):1922–9.
Longo FM, Massa SM. Small-molecule modulation of neurotrophin receptors: a strategy for the treatment of neurological disease. Nat Rev Drug Discov 2013;12(7):507–25.
Magnon C, Hall SJ, Lin J, Xue X, Gerber L, Freedland SJ, et al. Autonomic nerve development contributes to prostate cancer progression. Science 2013;341(6142):1236361.
Maisonpierre PC, Le Beau MM, Espinosa 3rd R, Ip NY, Belluscio L, de la Monte SM, et al. Human and rat brain-derived neurotrophic factor and neurotrophin-3: gene structures, distributions, and chromosomal localizations. Genomics 1991;10(3): 558–68.
McCaffrey G, Thompson ML, Majuta L, Fealk MN, Chartier S, Longo G, et al. NGF blockade at early times during bone cancer development attenuates bone destruction and increases limb use. Cancer Res 2014;74(23):7014–23.
Nilsson G, Forsberg-Nilsson K, Xiang Z, Hallbook F, Nilsson K, Metcalfe DD. Human mast cells express functional TrkA and are a source of nerve growth factor. Eur J Immunol 1997;27(9):2295–301.
Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P, et al. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 2006;127(3):635–48.
Pawson T. Regulation and targets of receptor tyrosine kinases. Eur J Cancer 2002;38(Suppl. 5):S3–10.

Pezet S, McMahon SB. Neurotrophins: mediators and modulators of pain. Annu Rev Neurosci 2006;29:507–38.
Pundavela J, Demont Y, Jobling P, Lincz LF, Roselli S, Thorne R, et al. ProNGF correlates with gleason score and is a potential driver of nerve infiltration in prostate cancer. Am J Pathol 2014 (in press).
Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM. Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 1987;325(6105):593–7.
Raffioni S, Bradshaw RA, Buxser SE. The receptors for nerve growth factor and other neurotrophins. Annu Rev Biochem 1993;62:823–50.
Reichardt LF. Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci 2006;361(1473):1545–64.
Romon R, Adriaenssens E, Lagadec C, Germain E, Hondermarck H, Le Bourhis X. Nerve growth factor promotes breast cancer angiogenesis by activating multiple pathways. Mol Cancer 2010;9:157.
Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell 2000;103(2):211–25.
Scott J, Selby M, Urdea M, Quiroga M, Bell GI, Rutter WJ. Isolation and nucleotide sequence of a cDNA encoding the precursor of mouse nerve growth factor. Nature 1983;302(5908):538–40.
Seidah NG, Benjannet S, Pareek S, Chretien M, Murphy RA. Cellular processing of the neurotrophin precursors of NT3 and BDNF by the mammalian proprotein convertases. FEBS Lett 1996;379(3):247–50.
Shooter EM. Early days of the nerve growth factor proteins. Annu Rev Neurosci 2001;24:601–29.
Swartz MA, Iida N, Roberts EW, Sangaletti S, Wong MH, Yull FE, et al. Tumor microenvironment complexity: emerging roles in cancer therapy. Cancer Res 2012;72(10):2473–80.
Teng KK, Felice S, Kim T, Hempstead BL. Understanding proneurotrophin actions: recent advances and challenges. Dev Neurobiol 2010;70(5):350–9.
Tomita T. Localization of nerve fibers in colonic polyps, adenomas, and adenocarcinomas by immunocytochemical staining for PGP 9.5. Dig Dis Sci 2012;57(2):364–70.
Truzzi F, Marconi A, Lotti R, Dallaglio K, French LE, Hempstead BL, et al. Neurotrophins and their receptors stimulate melanoma cell proliferation and migration. J Invest Dermatol 2008;128(8):2031–40.
Ullrich A, Gray A, Berman C, Dull TJ. Human beta-nerve growth factor gene sequence highly homologous to that of mouse. Nature 1983;303(5920):821–5.
Villers A, McNeal JE, Redwine EA, Freiha FS, Stamey TA. The role of perineural space invasion in the local spread of prostatic adenocarcinoma. J Urol 1989;142(3):763–8.
Zeidan Q, Hart GW. The intersections between O-GlcNAcylation and phosphorylation: implications for multiple signaling pathways. J Cell Sci 2010;123(Pt 1):13–22.
Zhao Q, Yang Y, Liang X, Du G, Liu L, Lu L, et al. The clinicopathological significance of neurogenesis in breast cancer. BMC Cancer 2014;14:484.

CHAPTER 3 PRONGF CORRELATES WITH GLEASON SCORE AND IS A POTENTIAL DRIVER OF NERVE INFILTRATION IN PROSTATE CANCER

3.1 PREFACE

It has been demonstrated in prostate cancer that development and ultimately infiltration of nerves involving the parasympathetic and sympathetic nervous system has a direct impact on tumor growth and dissemination. Strikingly, the presence of nerve fibers is directly correlated to aggressive types of prostate cancer − that is with high Gleason scores. Yet, the mechanism as to what drive nerves to infiltrate in prostate cancer is unknown. In order to address such knowledge gap, the current chapter shows that proNGF, detected in tumor microarrays by immunohistochemistry, is associated with prostate tumors with high Gleason scores. Furthermore, proNGF produced by prostate cancer cells induced axonogenesis of neurons in an *in vitro* co-culture model. In this study NGF was not detected readily in prostate cancer cells, suggesting that proNGF was the active form that stimulated axonogenesis. The report presented in this chapter is in the form of a published paper in *The American Journal of Pathology* entitled *"**ProNGF correlates with Gleason score and is a potential driver of nerve infiltration in prostate cancer**"*.

3.2 PUBLICATION

The American Journal of Pathology, Vol. 184, No. 12, December 2014

The American Journal of PATHOLOGY
ajp.amjpathol.org

SHORT COMMUNICATION

ProNGF Correlates with Gleason Score and Is a Potential Driver of Nerve Infiltration in Prostate Cancer

Jay Pundavela,[*†] Yohann Demont,[‡] Phillip Jobling,[*†] Lisa F. Lincz,[*§] Severine Roselli,[*†] Rick F. Thorne,[*†] Danielle Bond,[*†] Ralph A. Bradshaw,[¶] Marjorie M. Walker,[†‖] and Hubert Hondermarck[*†]

From the Schools of Biomedical Sciences and Pharmacy and Medicine and Public Health,[‖] Faculty of Health and Medicine, and the Hunter Medical Research Institute,[†] University of Newcastle, Callaghan, New South Wales, Australia; INSERM U908,[‡] University Lille 1, Villeneuve d'Ascq, France; the Hunter Haematology Research Group,[§] Calvary Mater Hospital, Waratah, New South Wales, Australia; and the Department of Pharmaceutical Chemistry,[¶] University of California, San Francisco, California*

Accepted for publication August 4, 2014.

Address correspondence to Hubert Hondermarck, Ph.D., Life Sciences Building (LS3-35), University of Newcastle, Callaghan, NSW 2308, Australia. E-mail: hubert.hondermarck@newcastle.edu.au.

Nerve infiltration is essential to prostate cancer progression, but the mechanism by which nerves are attracted to prostate tumors remains unknown. We report that the precursor of nerve growth factor (proNGF) is overexpressed in prostate cancer and involved in the ability of prostate cancer cells to induce axonogenesis. A series of 120 prostate cancer and benign prostate hyperplasia (BPH) samples were analyzed by IHC for proNGF. ProNGF was mainly localized in the cytoplasm of epithelial cells, with marked expression in cancer compared with BPH. Importantly, the proNGF level positively correlated with the Gleason score ($n = 104$, $\tau_B = 0.51$). A higher level of proNGF was observed in tumors with a Gleason score of ≥ 8 compared with a Gleason score of 7 and 6 ($P < 0.001$). *In vitro*, proNGF was detected in LNCaP, DU145, and PC-3 prostate cancer cells and BPH-1 cells but not in RWPE-1 immortalized nontumorigenic prostate epithelial cells or primary normal prostate epithelial cells. Co-culture of PC12 neuronal-like cells or 50B11 neurons with PC-3 cells resulted in neurite outgrowth in neuronal cells that was inhibited by blocking antibodies against proNGF, indicating that prostate cancer cells can induce axonogenesis via secretion of proNGF. These data reveal that ProNGF is a biomarker associated with high-risk prostate cancers and a potential driver of infiltration by nerves. *(Am J Pathol 2014, 184: 3156–3162; http://dx.doi.org/10.1016/j.ajpath.2014.08.009)*

A recent study[1] reported that autonomic nerve sprouting in prostate tumors contributes to prostate cancer progression. Sympathetic and parasympathetic nerve fibers were found to be necessary from initial to late phases of prostate cancer development. The density of nerve fibers in human prostate cancers was directly correlated to the Gleason prostate cancer score, and in an animal model, denervation resulted in a decrease in tumor engraftment and metastasis via a mechanism that involved the stimulation of β-adrenergic receptors on the surface of prostate cancer cells. This study is the first clear demonstration that the nervous system is involved in cancer progression and that nerve fibers are an essential component of the tumor microenvironment, participating in cancer growth and metastasis. However, the mechanisms by which nerve fibers are attracted to prostate tumors remain to be elucidated.

Nerve growth factor (NGF), the archetypal neurotrophin, is essential to the development of the peripheral and autonomic nervous systems because organs targeted by innervation produce NGF to stimulate neurite outgrowth and attract nerve fibers.[2] NGF drives the progressive innervation of the body and acts on neurons through the membrane tyrosine kinase receptor TrkA and the death receptor $p75^{NTR}$, activating various signaling pathways, including the mitogen-activated protein kinases, phosphatidylinositol 3-kinase, and NF-κB. The production of NGF is made through the synthesis of a biochemical precursor, proNGF, which can be cleaved either intracellularly by the protease furin or extracellularly by

http://dx.doi.org/10.1016/j.ajpath.2014.08.009

Supported by funding from the University of Newcastle, Australia, and the Hunter Translational Cancer Research Alliance.

Disclosures: H.H. and Y.D. are inventors on patent US20130171173 A1, method for proNGF assay for *in vitro* diagnosis of cancer in particular breast, thyroid, or lung cancer and therapeutic use of proNGF.

Current address of Y.D., INSERM U872, Centre de Recherche des Cordeliers, Paris, France.

metalloproteases to produce mature NGF.[3] Thus, proNGF is a reservoir of mature NGF, and the level of proNGF in a tissue reflects *NGF* gene expression. Interestingly, proNGF per se is also a biologically active molecule, initially described as an inducer of neuron apoptosis through interaction with sortilin and $p75^{NTR}$.[4] However, recent studies have also described its neurotrophic activities (ie, its ability to induce axonogenesis), and proNGF can stimulate neuron survival and differentiation by direct interaction with TrkA and $p75^{NTR}$, resulting in the activation of the mitogen-activated protein kinase pathway.[5–9]

In prostate cancer cells, a high-molecular-weight anti-NGF reactive protein, presumably corresponding to proNGF, has been described.[10] Therefore, by analogy with embryonic and postnatal development, it could be hypothesized that proNGF expression participates in attracting nerve fibers in prostate cancers. However, the production of proNGF has not been reported in a large cohort of prostate cancers, and it is not known whether it correlates with cancer aggressiveness. We explored the level of proNGF in such a cohort of prostate cancers and investigated a possible correlation with Gleason score. In addition, we co-cultured prostate cancer cells with neuronal cells to test the hypothesis that proNGF production by prostate cancer cells could stimulate axonogenesis.

Material and Methods

Prostate Tissue Samples and Cell Lines

High-density tumor microarrays of prostate cancers and adjacent benign prostatic hyperplasia (BPH) (PR802 and PR951) were obtained from Biomax (Rockville, MD). This cohort contained a large proportion of high-grade prostatic cancers, and information on treatment of patients was not available. Primary prostate epithelial cells (PrECs) were purchased from Lonza (Walkersville, MD). LNCaP cells (extracted from a lymph node metastasis of prostate cancer) were purchased from the ATCC (Manassas, VA) (CRL-1740). PC-3 prostate cancer cells (ATCC CRL-1435, derived from a bone metastasis of prostate cancer), BPH-1 cells (from a patient with BPH), DU145 cells (ATCC HTB-81, derived from a brain metastasis of prostate cancer), and RWPE-1 transformed nontumorigenic prostate epithelial cells were gifts from Dr. Judith Weidenhofer (University of Newcastle, NSW, Australia). The neuronal-like PC12 cells (ATCC CRL-1721) were from Prof. Ralph A. Bradshaw (University of California, San Francisco, CA). Immortalized dorsal root ganglia neurons 50B11 were obtained from Dr. Ahmet Höke (John Hopkins University, Baltimore, MD). PrECs were cultured in prostate primary epithelial cell culture media supplemented with PrEGM SingleQuots (Lonza, Walkersville, MD). BPH-1, RWPE-1, LNCaP, PC-3, and DU145 cells were cultured in RPMI 1640 supplemented with 2 mmol/L L-glutamine and 10% fetal calf serum (JRH Biosciences, Lenexa, KS). PC12 cells were maintained in Dulbecco's modified eagle medium from Life Technologies (Victoria, Australia) with 5% fetal calf serum, 10% horse serum (Sigma, South Australia, Australia), and 2 mmol/L L-glutamine. 50B11 neurons were maintained in neurobasal-A medium (Invitrogen, Victoria, Australia) with 10% fetal calf serum. All cell lines were grown in 75 cm^2 tissue culture flasks in a humidified incubator at 37°C with 5% CO_2.

Immunohistochemistry

After deparaffinization and hydration of tumor microarrays, antigens were retrieved at 60°C in 10 mmol/L citrate (pH 6) buffer. Endogenous peroxidases were quenched by immerging slides in TBS-Tween 0.1%, containing 3% H_2O_2 (10 minutes at room temperature), and saturation in Tris-buffered saline and Tween 0.1% with 3% bovine serum albumin (60 minutes, 37°C) was performed. Slides were probed with anti-proNGF antibody (AB9040 from Millipore, Billerica, MA) or normal rabbit IgG (AB-105-C from R&D Systems, Minneapolis, MN) as a negative control, at 1/200 in saturating buffer for 2 hours at 37°C. The signal was amplified with horseradish peroxidase–conjugated antibodies 711-035-152 anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) 1/400 in saturating buffer for 2 hours at 37°C. Immunostaining was visualized with diaminobenzidine chromogen (Sigma-Aldrich, St. Louis, MO), and slides were poststained with Harris hematoxylin and mounted before observation with a Nikon Eclipse Ti-U microscope.

Double Immunostaining

Stainings were performed sequentially using ImmPRESS reagents as per the manufacturer's recommendations (Vector Laboratories, Burlingame, CA). Briefly, after deparaffinization, rehydration, inactivation of endogenous peroxidases with H_2O_2, and blocking with 2.5% horse serum, the mouse proNGF was first applied to the sections followed by the ImmPRESS horseradish peroxidase anti-mouse IgG (peroxidase) and incubation with diaminobenzidine peroxidase substrate solution (Vector Laboratories). Then the slides were washed for 5 minutes in phosphate-buffered saline, blocked, stained with rabbit PGP9.5 (Abcam, Cambridge, MA) antibody, and incubated with the ImmPRESS alkaline phosphatase anti-rabbit IgG before incubation with Vector Red alkaline phosphatase substrate (Vector Laboratories). Finally, tissue microarray slides were counterstained with hematoxylin (Supplemental Figure S1).

Quantification of proNGF Labeling and Analysis of Correlation with Gleason Score

Determination of prostate tumor Gleason score according to the International Society of Urological Pathology 2005 modified Gleason grading system[11] was performed by a histopathologist (M.M.W.). Tissues used in the present study all had architectural patterns considered to be Gleason

grade 3, 4, or 5 and were categorized as a Gleason score of 6 (3+3), a Gleason score of 7 (3+4 and 4+3), and a Gleason score of ≥8 (4+4, 4+5, 5+4, 5+5). Two independent observers estimated the intensity of anti-proNGF staining as 0 (no staining), 1 (weak staining), 2 (medium staining), and 3 (intense staining), as described previously.[12] For association of proNGF staining intensity with Gleason grade (3, 4, or 5), a total of 264 tissue areas from 120 patients (104 cancers + 16 cases of BPH) were available for analysis. Individual Gleason grade and overall Gleason score (the sum of the two most represented Gleason grade areas per patient) was compared with proNGF staining intensity and analyzed as ordinal variables using χ^2 or Fisher's exact tests, with Kendall's τ_B rank correlation coefficient calculated as a measure of association. Analysis was performed using Statistica statistical software version 10 (StatSoft Inc., Tulsa, OK), and STATA statistical software version 11 (StataCorp, College Station, TX).

Digital Analysis of proNGF Labeling

Ten representative regions of interest were defined for each image, highlighting epithelial areas. Diaminobenzidine staining was revealed using the Color Deconvolution algorithm (version 9.1) before applying the Positive Pixel Count algorithm (version 9.1) to obtain the mean intensity of all pixels in each region of interest. Nuclei in each region of interest were counted, and a cell density index was calculated as number of cells per square millimeter. Box and whisker plots were generated from the data using BoxPlotR software (interactive web application running in R version 3.1 using Shiny version 1.2) (Supplemental Figure S2).

Protein Extraction and Western Blotting

Subconfluent cell monolayers were lyzed with 1% NP40 lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP40 pH 8.0) that contained complete EDTA free protease inhibitor cocktail (Roche, Mannheim, Germany). Insoluble proteins were removed by centrifugation at $15 \times 10^3 \times g$ for 10 minutes (at 4°C), and the total protein concentration was determined using the micro BCA kit (Pierce Biotechnology, Rockford, IL) per the manufacturer's instructions. Next 20-μg proteins were separated by SDS-PAGE with 12% resolving gel and then transferred to 0.4-μm pore nitrocellulose membranes (Amersham, GE Healthcare Life Sciences, Pittsburgh, PA) using a wet transblotter (BioRad, Gladesville, NSW, Australia). Blots were blocked with blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 hour at room temperature and then probed with antibodies against proNGF (1/500; AB9040 from Millipore) and β-actin (1/5000; Sigma-Aldrich) diluted in the blocking buffer. After washing with phosphate-buffered saline that contained 0.1% Tween 20, membranes were probed with goat anti-mouse or goat anti-rabbit IR-Dye 670 or 800cw–labeled secondary antisera then washes were repeated after labeling. Western blot was imaged using the LI-COR Odyssey infrared imaging system (LI-COR) and densitometric analysis of immunoreactive bands was determined using the Image Studio Lite (LI-COR).

Co-Cultures and Neurite Outgrowth Assay

For co-culture experiments, PC-12 cells (5×10^4 in 1 mL) were seeded in the lower compartment of Transwell plates (Corning Inc., Midland, MI) coated with rattail collagen I (Invitrogen). After 24 hours, cells were serum starved in Dulbecco's modified eagle medium that contained 1% horse serum. Co-cultures were performed with PC-3 prostate cancer cells grown in the upper Transwell inserts (12.0 mm in diameter with 3.0-μm pores) (Corning Inc.) with or without anti-proNGF blocking monoclonal antibodies (Alomone, Jerusalem, Israel). Control was performed with isotype antibody. Differentiation of PC12 cells was allowed for 4 days, and neurite outgrowth was measured per square millimeter. PC12 cells were considered differentiated when they exhibited neurites of at least twice the length of the cell body. For co-culture with the 50B11 cells, the same protocol was applied, but the culture media included 5 μmol/L

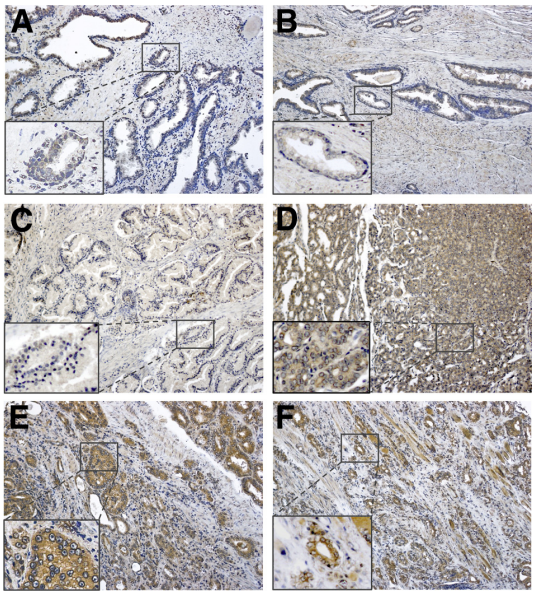


Figure 1 Immunohistochemical detection of precursor of nerve growth factor (proNGF). ProNGF was immunodetected in a series of 120 prostate cancers and benign prostate hyperplasia. Representative images are shown. **A** and **B:** Benign prostate hyperplasia (no staining in epithelial cells, 0). **C:** Gleason score 3+3 cancer (low intensity staining, 1). **D:** Gleason score 4+4 cancer (high intensity staining, 3). **E:** Gleason score 4+5 cancer (high intensity staining, 3). **F:** Gleason score 4+4 cancer (medium intensity staining, 2). **Boxed areas** show higher magnification of proNGF cellular staining. Quantitative image analyses of the tumors presented here are shown in Supplemental Figure S2. Prostate cancers and benign prostate hyperplasia (n = 120) were analyzed. Original magnification: ×100 (**A**–**F**); ×400 (**boxed areas, A**–**F**).

forskolin (necessary to obtain neurite outgrowth with these cells). Images were obtained using an inverted microscope (Zeiss, Jena, Germany). One-way analysis of variance statistical test (GraphPad Prism version 5.01; GraphPad Software, La Jolla, CA) was used.

Results

ProNGF Level Correlates with Gleason Score

Immunohistochemistry (IHC) of cancer tissue samples revealed the presence of proNGF mainly in the cytoplasm of epithelial cancer cells, with stromal cells exhibiting a low level of staining (Figure 1). Absence of proNGF staining was observed in 13% of BPH tissues (Figure 1, A and B), whereas various levels of proNGF could be observed among cancers of different Gleason scores (Figure 1, C–F). Digital quantification (Supplemental Figure S2) indicated an increase in proNGF (regardless of cell density) in Figure 1, C–F (cancer), compared with Figure 1, A and B (BPH). This increase of proNGF on a per cell basis is also visible in enlargement boxes for each panel. The frequency distribution of proNGF levels (Figure 2A) revealed that most BPH and Gleason score 6 cancers had low levels of proNGF (staining intensity, 0 and 1), whereas the proportion of cases with intermediate (staining intensity, 2) and high (staining intensity, 3) levels of proNGF increased in Gleason score 7 and ≥8 cancers ($P < 0.001$). Therefore, most cases with the highest Gleason score exhibited the most intense proNGF labeling levels. Highest levels of proNGF (staining intensity, 3) were observed only in Gleason score 7 and ≥8 cancers and not in Gleason score 6 cancers. We also looked at proNGF level in individual Gleason grade areas (Gleason score being the sum of the two most abundant Gleason grades observed in a section) (Figure 2B). The proportion of cases with intermediate (staining intensity, 2) and high (staining intensity, 3) levels of proNGF increased in Gleason grade areas of 4 and 5 compared with Gleason grade 3 areas ($P < 0.0005$). Together, there was a positive correlation between proNGF

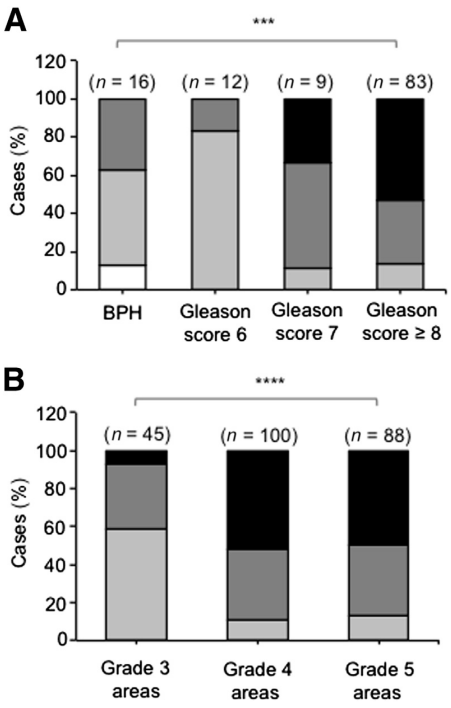


Figure 2 Frequency distribution of precursor of nerve growth factor (proNGF) level according to Gleason score. ProNGF levels [0, no staining (white); 1, low intensity staining (light gray); 2, intermediate intensity staining (dark gray); and 3, high intensity staining (black)] were measured in epithelial cells of benign prostatic hyperplasia (BPH) and prostate cancers. **A:** Distribution according to Gleason score. Tumors with high Gleason scores of 7 and ≥8 (3+4, 4+3, 4+5, 5+4, 5+5) were more likely to have high proNGF expression than tumors with low Gleason scores (Gleason grades 3+3) and BPH. The calculated coefficient of correlation between proNGF staining intensity and Gleason score was $\tau_B = 0.51$. **B:** Distribution according to Gleason grade. The proportion of tissue areas expressing high levels of ProNGF increased in Gleason grade 4 and 5 areas compared with Gleason grade 3 areas. ***$P < 0.001$, Fisher's exact test; ****$P < 0.0005$, χ^2 test.

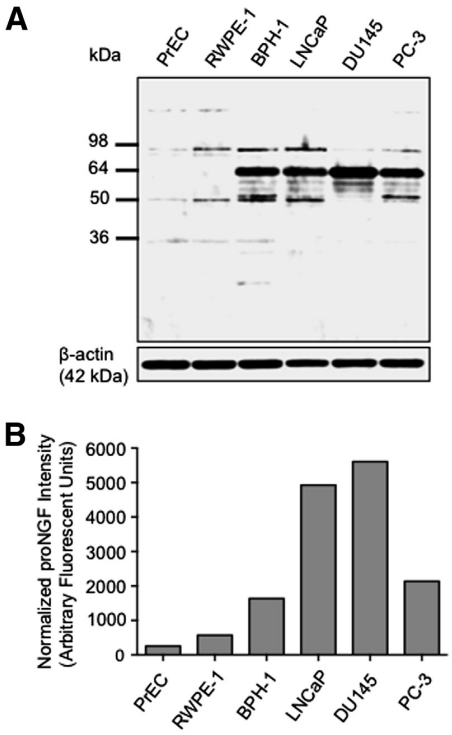


Figure 3 Precursor of nerve growth factor (proNGF) production in prostate cancer cell lines. **A:** Western blot detects proNGF in normal prostate epithelial cells (PrECs), transformed nontumorigenic prostate epithelial cells (RWPE-1), benign prostate hyperplasia cells (BPH-1), and prostate cancer cells (LNCaP, DU415, PC-3). A major band is observed at 60 kDa and two other minor bands at 50 and 85 kDa. **B:** Densitometric quantification of proNGF. Normalization was performed relative to β-actin.

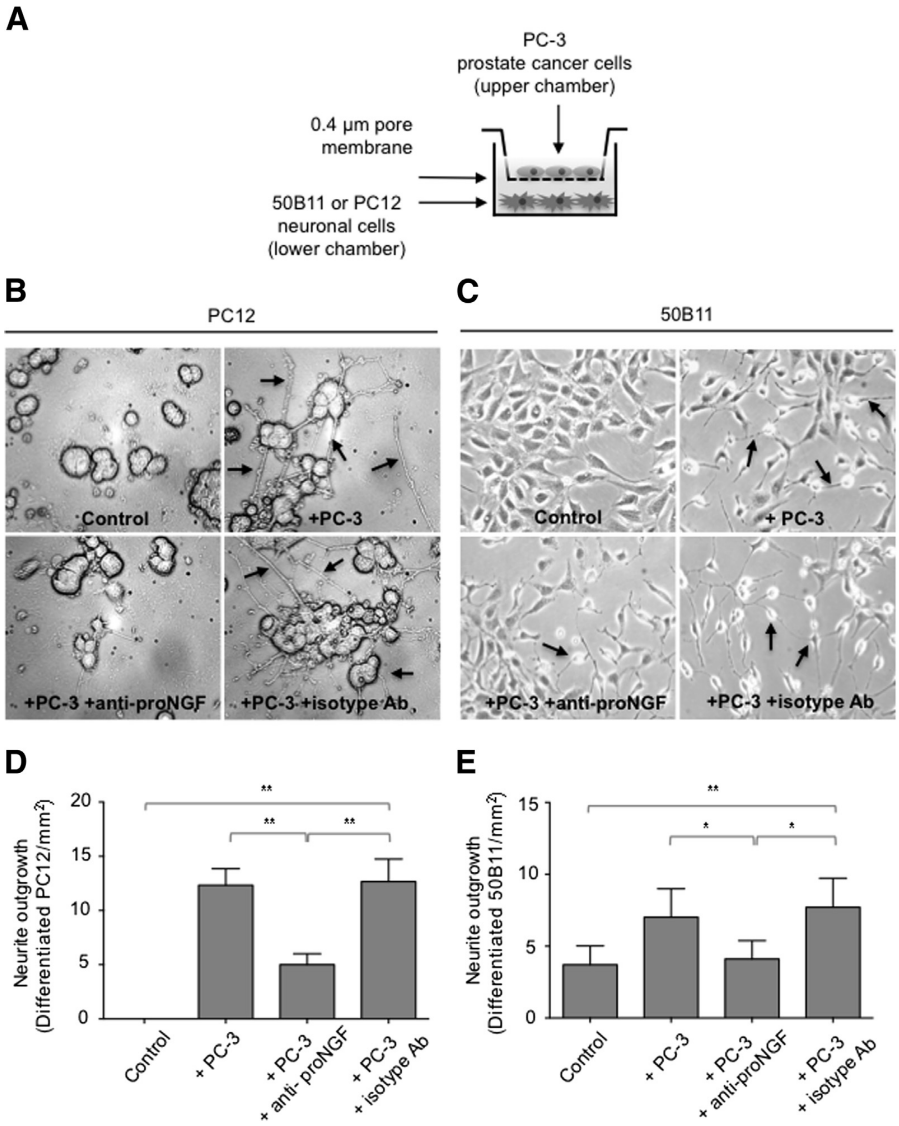


Figure 4 Axonogenesis is induced by prostate cancer cells. **A:** Co-culture experiments were performed in Transwell Boyden chambers with PC-3 prostate cancer cells in the upper chamber and neuronal cells (PC12 or 50B11) in the lower chamber. Neurite outgrowth started after 3 days. **B** and **C:** After 4 days of co-culture, PC12 and 50B11 cells remain undifferentiated in the absence of PC-3 cells (control), and neurite outgrowth is observed in co-culture with PC-3 (+PC-3). This neurotrophic effect is diminished in the presence of anti-proNGF blocking antibodies (+PC-3 + anti-proNGF antibody), whereas the isotype control antibodies (+PC-3 + isotype antibody) do not inhibit axonogenesis. **Arrows** indicate neurites. **D** and **E:** Quantification of neurite outgrowth in PC12 and 50B11 cells. Experiments were performed in triplicate and data represent means ± SD. Analysis of variance test was used. $*P < 0.05$, $**P < 0.01$.

intensity levels and Gleason grades ($n = 264$, coefficient of correlation $\tau_B = 0.37$) that became more pronounced when proNGF intensity levels were considered against Gleason scores ($n = 104$, coefficient of correlation $\tau_B = 0.51$). This pattern of proNGF relative to Gleason score was similar to the nerve infiltration described by Magnon et al[1] because they found that nerve infiltration correlated with Gleason score. Although we have not investigated nerve infiltration in the present study, we have been able to co-localize proNGF with nerve fibers (Supplemental Figure S1). Together, these data prompted us to look for a proNGF-mediated ability of prostate cancer cells to stimulate axonogenesis.

ProNGF Production in Prostate Cancer Cell Lines

Western blot analysis of a panel of normal and cancer prostate cells indicated a major immunoreactive band at 60 kDa (Figure 3A). On the basis of amino acid sequence, the

theoretical molecular mass of proNGF is 26 kDa, but there are two glycosylation sites, and various apparent molecular masses up to 100 kDa have been reported, including 60 kDa in the uterus.[13] Interestingly, Djakiew et al[14] used Western blotting with an anti-NGF antibody in prostatic cancer to describe an immunoreactive band of approximately 60 kDa that they called the NGF-like protein. This study was performed before the discovery of proNGF, and our results, confirming the molecular weight of 60 kDa, indicate that it was actually proNGF. Densitometric analysis of the Western blotting (Figure 3B) revealed that proNGF level was minimal in primary PrECs and RWPE-1 immortalized nontumorigenic epithelial cells, increased in the BPH-1 benign prostate hyperplasia cells, and higher in LNCaP, DU145, and PC-3 cancer cells. These data mirror the IHC analyses (Figures 1 and 2) indicating a higher level of proNGF in prostatic cancer cells compared with those from BPH. In addition, mature NGF was not detected in any of the prostate cells tested (data not shown), suggesting that proNGF is not processed to mature NGF in prostate cancer cells.

Neurotrophic Activity of Prostate Cancer Cells Involves proNGF

Co-culture experiments in Transwell Boyden chambers were performed between PC-3 prostate cancer cells and PC12 neuronal-like cells or the 50B11 immortalized dorsal root ganglia neurons (Figure 4A). The results revealed that PC-3 cells could induce neuronal differentiation of PC12 cells (Figure 4B) and 50B11 cells (Figure 4C). Neurite outgrowth was observed when PC12 cells or 50B11 cells were co-cultured with PC-3, whereas no differentiation occurred in the control situation in which the neuronal cells were grown alone. Interestingly, blocking antibodies against proNGF diminished PC-3–induced differentiation of PC12 and 50B11 cells (Figure 4, B and C). Less differentiated cells and shorter neurites were observed in the presence of the anti-proNGF antibodies. The number of differentiated cells was quantified for PC12 and 50B11 (Figures 4D and 4E). Together these data reveal that prostate cancer cells are able to induce axonogenesis through the production of proNGF.

Discussion

The level of proNGF that we describe here in relation to Gleason score in prostate tumors is comparable to the association between Gleason score and the density of nerve infiltration reported by Magnon et al.[1] They found a higher density of nerve fibers in cancers of high Gleason score. However, they did not suggest an explanation for the mechanism of nerve infiltration. Although we have no human data on axonogenesis, our observation that proNGF is increased in cancers of high Gleason score points to proNGF overexpression by prostate cancer cells as a possible explanation for nerve infiltration in prostate cancer. By using co-culture with PC12 cells and 50B11 neurons, we found that prostate cancer cells can induce neuronal differentiation and axonogenesis by a mechanism that involves the production of proNGF. PC12 cells are not true neurons, but they are the canonical *in vitro* model for studying neurotrophic factors and their signaling pathways. Membrane receptors for NGF and proNGF, TrkA-$p75^{NTR}$-sortilin are expressed in PC12 cells and mediate NGF-stimulated induction of neurodifferentiation. It has been found that proNGF can induce differentiation of PC12 cells even though it is unclear whether proNGF acts directly or through the generation of mature NGF because it depends on the relative levels of its receptors TrkA and $p75^{NTR}$[7] and the intracellular cleavage of proNGF into mature NGF.[8,15] In the present work, NGF could not be detected in prostate cancer cells and conditioned media (data not shown), which therefore suggested that proNGF itself was responsible for axonogenesis. Overall, these data indicated that the production of proNGF by cancer cells is a potential driver of nerve infiltration in prostate cancer.

Prostatic cancer cells have been described to respond to NGF stimulation by increasing proliferation and migration via the activation of the membrane receptors TrkA and $p75^{NTR}$,[16] and changes in the expression of these ligands/receptors contribute to prostate tumor cell growth and dissemination through mitogen-activated protein kinase–regulated signaling pathways.[17,18] In addition, inhibition of the Trk receptor axis decreases the growth of prostatic cancer cell xenografts in nude mice,[19] indicating that the proNGF/NGF/receptors axis is active in prostate cancer. Our results reveal that a neurotrophic activity of proNGF, produced by prostate cancer cells, is also to be taken into consideration for its effect on tumor progression. From a therapeutic perspective, the study by Magnon et al[1] found that targeting nerve fibers in prostate cancer can inhibit cancer growth and metastasis. However, the drug used (6-hydroxydopamine) is unlikely to be of clinical use to treat prostate cancer because it crosses the blood brain barrier and is highly toxic for the central nervous system.[20] Therefore, identifying the cause of nerve infiltration in prostate cancer and finding ways to block nerve infiltration without inducing neuronal toxicity is of great importance to any future translation to the clinic. The identification of proNGF as a driver of prostate cancer innervation offers a rationale for testing the therapeutic potential of targeting this polypeptide growth factor in prostate cancer.

NGF is a mediator of pain, and its receptors TrkA and $p75^{NTR}$ are nociceptors whose activation in sensory neurons results in the transmission of the feeling of pain to the central nervous system.[21,22] Interestingly, it has been found in a murine model that anti-NGF antibody (which can also target proNGF) decreases pain induced by bone metastasis of prostate cancer cells,[23,24] and a humanized monoclonal antibody (Tanezumab) has already entered clinical trials for its analgesic activity in chronic and acute pain.[25] Therefore, targeting proNGF could also have an additional positive effect in prostate cancer by reducing metastasis-induced pain.

In conclusion, we found that proNGF is overexpressed in human prostate cancer, it correlates with Gleason score, and

its production by cancer cells can potentially drive axonogenesis. Thus, the value of proNGF as a clinical biomarker for prognosis and as a therapeutic target to inhibit nerve infiltration in prostatic cancer should be further investigated.

Acknowledgments

We thank Sheridan Keene for excellent technical assistance and Benoni Boilly (University of Lille) for critical reading of the manuscript.

Y.D. performed the IHC; tissue slide analysis, grading, and scoring were performed by M.M.W. and confirmed by H.H. and R.T.; J.P. performed the *in vitro* experiments and prepared all figures; P.J. contributed to neuronal cell cultures; L.L. performed statistical analyses; D.B. contributed to cell culture and protein extraction; H.H. conceived and supervised the study with significant input from S.R. and R.A.B.; H.H. and J.P. drafted the manuscript, which was read and approved by all co-authors.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2014.08.009*.

References

1. Magnon C, Hall SJ, Lin J, Xue X, Gerber L, Freedland SJ, Frenette PS: Autonomic nerve development contributes to prostate cancer progression. Science 2013, 341:1236361
2. Chao MV: Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci 2003, 4:299–309
3. Lee R, Kermani P, Teng KK, Hempstead BL: Regulation of cell survival by secreted proneurotrophins. Science 2001, 294:1945–1948
4. Nykjaer A, Lee R, Teng KK, Jansen P, Madsen P, Nielsen MS, Schwarz E, Willnow TE, Hempstead BL, Petersen CM: Sortilin is essential for proNGF-induced neuronal cell death. Nature 2004, 427:843–848
5. Fahnestock M, Yu G, Michalski B, Mathew S, Colquhoun A, Ross GM, Coughlin MD: The nerve growth factor precursor proNGF exhibits neurotrophic activity but is less active than mature nerve growth factor. J Neurochem 2004, 89:581–592
6. Clewes O, Fahey MS, Tyler SJ, Watson JJ, Seok H, Catania C, Cho K, Dawbarn D, Allen SJ: Human ProNGF: biological effects and binding profiles at TrkA, P75NTR and sortilin. J Neurochem 2008, 107:1124–1135
7. Masoudi R, Ioannou MS, Coughlin MD, Pagadala P, Neet KE, Clewes O, Allen SJ, Dawbarn D, Fahnestock M: Biological activity of nerve growth factor precursor is dependent upon relative levels of its receptors. J Biol Chem 2009, 284:18424–18433
8. Kalous A, Nangle MR, Anastasia A, Hempstead BL, Keast JR: Neurotrophic actions initiated by proNGF in adult sensory neurons may require peri-somatic glia to drive local cleavage to NGF. J Neurochem 2012, 122:523–536
9. Howard L, Wyatt S, Nagappan G, Davies AM: ProNGF promotes neurite growth from a subset of NGF-dependent neurons by a p75NTR-dependent mechanism. Development 2013, 140:2108–2117
10. Delsite R, Djakiew D: Characterization of nerve growth factor precursor protein expression by human prostate stromal cells: a role in selective neurotrophin stimulation of prostate epithelial cell growth. Prostate 1999, 41:39–48
11. Epstein JI, Allsbrook WC Jr, Amin MB, Egevad LL: The 2005 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma. Am J Surg Pathol 2005, 29:1228–1242
12. Adriaenssens E, Vanhecke E, Saule P, Mougel A, Page A, Romon R, Nurcombe V, Le Bourhis X, Hondermarck H: Nerve growth factor is a potential therapeutic target in breast cancer. Cancer Res 2008, 68:346–351
13. Lobos E, Gebhardt C, Kluge A, Spanel-Borowski K: Expression of nerve growth factor (NGF) isoforms in the rat uterus during pregnancy: accumulation of precursor proNGF. Endocrinology 2005, 146:1922–1929
14. Djakiew D, Delsite R, Pflug B, Wrathall J, Lynch JH, Onoda M: Regulation of growth by a nerve growth factor-like protein which modulates paracrine interactions between a neoplastic epithelial cell line and stromal cells of the human prostate. Cancer Res 1991, 51:3304–3310
15. Boutilier J, Ceni C, Pagdala PC, Forgie A, Neet KE, Barker PA: Proneurotrophins require endocytosis and intracellular proteolysis to induce TrkA activation. J Biol Chem 2008, 283:12709–12716
16. Djakiew D, Pflug BR, Delsite R, Onoda M, Lynch JH, Arand G, Thompson EW: Chemotaxis and chemokinesis of human prostate tumor cell lines in response to human prostate stromal cell secretory proteins containing a nerve growth factor-like protein. Cancer Res 1993, 53:1416–1420
17. Sortino MA, Condorelli F, Vancheri C, Chiarenza A, Bernardini R, Consoli U, Consoli U, Canonico PL: Mitogenic effect of nerve growth factor (NGF) in LNCaP prostate adenocarcinoma cells: role of the high- and low-affinity NGF receptors. Mol Endocrinol 2000, 14:124–136
18. Weeraratna AT, Dalrymple SL, Lamb JC, Denmeade SR, Miknyoczki S, Dionne CA, Isaacs JT: Pan-trk inhibition decreases metastasis and enhances host survival in experimental models as a result of its selective induction of apoptosis of prostate cancer cells. Clin Cancer Res 2001, 7:2237–2245
19. Miknyoczki SJ, Wan W, Chang H, Dobrzanski P, Ruggeri BA, Dionne CA, Buchkovich K: The neurotrophin-trk receptor axes are critical for the growth and progression of human prostatic carcinoma and pancreatic ductal adenocarcinoma xenografts in nude mice. Clin Cancer Res 2002, 8:1924–1931
20. Glinka Y, Gassen M, Youdim MB: Mechanism of 6-hydroxydopamine neurotoxicity. J Neural Transm Suppl 1997, 50:55–66
21. Pezet S, McMahon SB: Neurotrophins: mediators and modulators of pain. Annu Rev Neurosci 2006, 29:507–538
22. Schmidt BL, Hamamoto DT, Simone DA, Wilcox GL: Mechanism of cancer pain. Mol Interv 2010, 10:164–178
23. Halvorson KG, Kubota K, Sevcik MA, Lindsay TH, Sotillo JE, Ghilardi JR, Rosol TJ, Boustany L, Shelton DL, Mantyh PW: A blocking antibody to nerve growth factor attenuates skeletal pain induced by prostate tumor cells growing in bone. Cancer Res 2005, 65:9426–9435
24. Jimenez-Andrade JM, Ghilardi JR, Castaneda-Corral G, Kuskowski MA, Mantyh PW: Preventive or late administration of anti-NGF therapy attenuates tumor-induced nerve sprouting, neuroma formation, and cancer pain. Pain 2011, 152:2564–2574
25. Cattaneo A: Tanezumab, a recombinant humanized mAb against nerve growth factor for the treatment of acute and chronic pain. Curr Opin Mol Ther 2010, 12:94–106

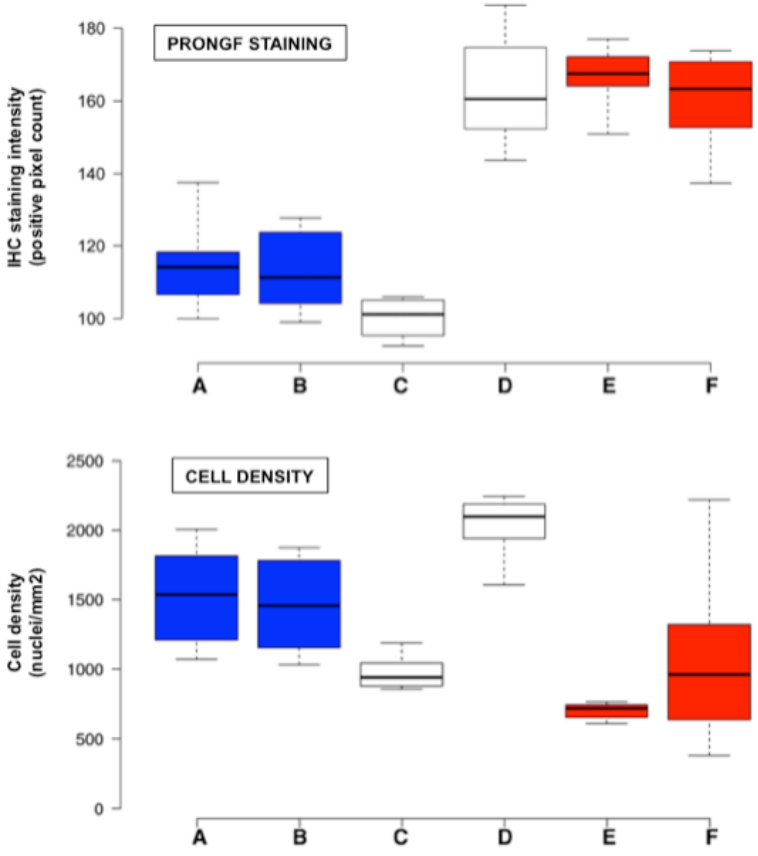
3.3 SUPPLEMENTAL FILES

SUPPLEMENTAL FIGURE LEGENDS

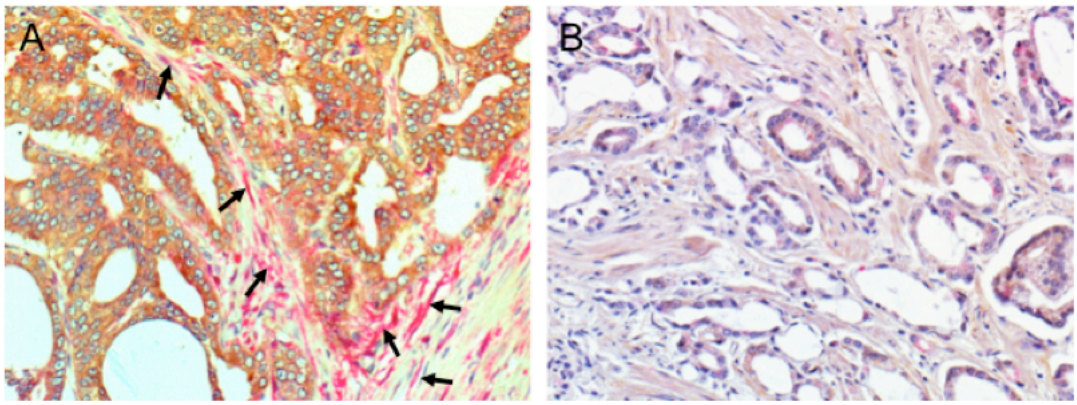
Figure S1. Digital analysis of proNGF labelling *versus* cell density of panels presented in Fig. 1. Specimens in Fig. 1 are representative of benign prostate hyperplasia (A and B), Gleason 3+3 cancer (C), Gleason 4+4 cancer (D), Gleason 4+5 cancer (E) and Gleason 4+4 cancer (F). Quantification of proNGF staining (**top graph**) indicated a higher proNGF level in D, E, F compared to A, B, C. Cell density measurements (**bottom graph**) showed similar number of cells per unit area between all panels (A to F). Therefore, on a per cell basis there is more proNGF staining in D, E, F (prostate cancer of high Gleason score) than in C (low Gleason score) and A, B (BPH). **Methods:** Digital images from Figure 1 were analysed using the Aperio ePathology system (Spectrum Version 11.2.0.780). Ten representative regions of interest (ROI) were defined for each image highlighting epithelial areas. DAB staining was revealed using the Color Deconvolution algorithm (v9.1) before applying the Positive Pixel Count algorithm (v9.1) to obtain the average intensity of all pixels (Iavg) in each ROI. Nuclei in each ROI were counted and a cell density index was calculated as cell number/mm^2. Box and Whisker plots were generated from the data using BoxPlotR software. Centre lines show the median value, boxes indicate the 25th and 75th percentiles and whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.

Figure S2. Co-localization between proNGF and nerve fibres. Double labelling was performed for proNGF (brown coloration) and the neuronal marker PGP9.5 (red coloration). Nerve fibres (in red and indicated by arrows) were observed in tumors with high level of proNGF (brown) **(A)** whereas they were not detected in tumors with low level of proNGF **(B)**.

Methods for the double immunolabeling. Stainings were performed sequentially using ImmPRESS reagents as per the manufacturers recommendations (Vector Laboratories). Briefly, after deparaffinization, rehydration, inactivation of endogenous peroxidases with H_2O_2, and blocking with 2.5% horse serum, the mouse proNGF was first applied to the sections followed by the ImmPRESS HPR anti-mouse IgG (peroxidase) and incubation with DAB peroxidase substrate solution (Vector laboratories). Then, the slides were washed for 5 min in PBS, blocked, stained with rabbit PGP9.5 (Abcam) antibody and incubated with the ImmPRESS AP anti-rabbit IgG (alkaline phosphatase) before incubation with Vector Red alkaline phosphatase substrate (Vector laboratories). Finally, TMA slides were counterstained with hematoxylin.



Supplemental Fig. S1, Pundavela et al.



Supplemental Fig. S2, Pundavela et al.

CHAPTER 4 NERVE FIBERS INFILTRATE THE TUMOR MICROENVIRONMENT AND ARE ASSOCIATED WITH NERVE GROWTH FACTOR PRODUCTION AND LYMPH NODE INVASION IN BREAST CANCER

4.1 PREFACE

Considering the results described in chapter 3, it was conceivable that a similar mechanism of nerve infiltration may occur in other solid tumors such as breast cancer in which proNGF/NGF are also overexpressed. It was first logical to examine if nerve fibers were present in breast cancer. In the current chapter the presence of nerve fibres was observed in aggressive invasive breast cancers. Moreover, a compelling observation is that NGF expression was directly associated with the presence of nerve fibers. In addition we demonstrated that NGF plays a role in the neurotrophic effect of breast cancer cells with a co-culture *in vitro* assay. The manuscript entitled *"**Nerve fibers infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer**"* presented has been accepted to the journal *Molecular Oncology*.

4.2 PUBLICATION

ARTICLE IN PRESS

MOLECULAR ONCOLOGY XXX (2015) 1–10

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/molonc

Nerve fibers infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer

Jay Pundavela[a,b,1], *Severine Roselli*[a,b,1], *Sam Faulkner*[a,b], *John Attia*[b,c], *Rodney J. Scott*[a,b], *Rick F. Thorne*[b], *John F. Forbes*[b,c], *Ralph A. Bradshaw*[d], *Marjorie M. Walker*[b,c], *Phillip Jobling*[a,b,1], *Hubert Hondermarck*[a,b,*,1]

[a]*School of Biomedical Sciences and Pharmacy, Faculty of Health and Medicine, University of Newcastle, Callaghan, NSW 2308, Australia*

[b]*Hunter Medical Research Institute, New Lambton Heights, NSW 2305, Australia*

[c]*School of Medicine and Public Health, Faculty of Health and Medicine, University of Newcastle, Callaghan, NSW 2308, Australia*

[d]*Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94158, USA*

ARTICLE INFO

Article history:
Received 18 March 2015
Received in revised form
1 May 2015
Accepted 4 May 2015
Available online ■

Keywords:
Nerve fibers (axons)
Tumor microenvironment
Nerve growth factor
Breast cancer

ABSTRACT

Infiltration of the tumor microenvironment by nerve fibers is an understudied aspect of breast carcinogenesis. In this study, the presence of nerve fibers was investigated in a cohort of 369 primary breast cancers (ductal carcinomas in situ, invasive ductal and lobular carcinomas) by immunohistochemistry for the neuronal marker PGP9.5. Isolated nerve fibers (axons) were detected in 28% of invasive ductal carcinomas as compared to only 12% of invasive lobular carcinomas and 8% of ductal carcinomas in situ ($p = 0.0003$). In invasive breast cancers, the presence of nerve fibers was observed in 15% of lymph node negative tumors and 28% of lymph node positive tumors ($p = 0.0031$), indicating a relationship with the metastatic potential. In addition, there was an association between the presence of nerve fibers and the expression of nerve growth factor (NGF) in cancer cells ($p = 0.0001$). *In vitro*, breast cancer cells were able to induce neurite outgrowth in PC12 cells, and this neurotrophic activity was partially inhibited by anti-NGF blocking antibodies. In conclusion, infiltration by nerve fibers is a feature of the tumor microenvironment that is associated with aggressiveness and involves NGF production by cancer cells. The potential participation of nerve fibers in breast cancer progression needs to be further considered.

Abbreviations: DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; EGF, epidermal growth factor; IHC, immunohistochemistry; NGF, nerve growth factor; PGP9.5, protein gene product 9.5; TMA, tumor microarrays.

* *Corresponding author*. School of Biomedical Sciences and Pharmacy, Life Sciences Building (LS3-35), University of Newcastle, Callaghan, NSW 2308, Australia. Tel.: +61 2492 18830; fax: +61 2492 16903.

E-mail address: hubert.hondermarck@newcastle.edu.au (H. Hondermarck).

[1] Contributed equally to this work.

http://dx.doi.org/10.1016/j.molonc.2015.05.001

1. Introduction

The role of the nervous system in cancer etiology, and in particular the influence of nerve fibers in the tumor microenvironment, is an understudied aspect of cancer biology (Ondicova and Mravec, 2010). It is well established that cancer cells can grow around existing nerves and eventually invade them in a process called perineural invasion (Marchesi et al., 2010). This is generally associated with a poor prognosis and can cause pain as demonstrated in pancreatic cancer (Bapat et al., 2011). Conversely, the infiltration of tumors by growing nerves, or tumor axonogenesis, has only recently been suggested to actively participate in cancer progression. Indeed, a recent report (Magnon et al., 2013) has revealed that tumor infiltration by nerve fibers is essential for prostate cancer progression from early initiation to metastasis. The mechanism remains unclear but includes liberation of catecholamines and acetylcholine in the vicinity of cancer cells, resulting in the stimulation of tumor growth and invasion. In addition, another recent study has shown that denervation suppresses gastric tumorigenesis (Zhao et al., 2014a), further pointing to the role of the nervous system in carcinogenesis. These pioneering studies in prostate and gastric cancer suggest a potential value of anti-neurogenic therapies (Jobling et al., 2015) and raise the possibility that tumor infiltration by nerve fibers may also be important in other types of cancer.

In breast cancer, little is known about nerve fibers in the tumor microenvironment. A study in mice has shown that nerve infiltration in bone metastases participates in the stimulation of metastatic growth (Campbell et al., 2012) and recent investigations have evidenced the presence of nerves in human primary breast tumors (Zhao et al., 2014b; Huang et al., 2014). Anatomical and histological studies have shown that the normal breast is innervated by both sympathetic and sensory fibers from the 4–6th thoracic nerves (Sarhadi et al., 1996). Sensory fibers supply the nipple and skin, whereas sympathetic fibers innervate blood vessels and ducts. Therefore, it is conceivable that nerve fibers could be attracted into breast tumors, especially in light of the evidence that breast cancer cells can produce neurotrophic growth factors such as the neurotrophins (Hondermarck, 2012). In particular, nerve growth factor (NGF), which can stimulate the growth of sympathetic and sensory nerves, is produced and secreted by breast cancer cells (Adriaenssens et al., 2008). During embryonic development, NGF plays a major role in directing nerves to their correct targets (Skaper, 2012) and similarly it can be hypothesized that the production of NGF by breast cancer cells may result in the attraction of nerve fibers into primary breast tumors.

In the present study, the hypothesis that nerve fibers are a significant component of the breast tumor microenvironment has been explored by analyzing a cohort of breast cancers. Nerve fibers were detected in a significant proportion of invasive breast cancers and their presence was associated with lymph node invasion, suggesting a relationship with metastatic potential. An association was found between the presence of nerve fibers and the expression of NGF by cancer cells, and in co-culture with neuronal cells, breast cancer cells were able to induce neuronal outgrowth via the release of NGF.

2. Materials and methods

2.1. Breast cancer tissue samples and cell lines

High-density tumor microarrays (TMAs) of invasive ductal carcinomas (IDC), invasive lobular carcinomas (ILC) and ductal carcinomas in situ (DCIS) of the breast were obtained from Biomax (Maryland, USA, catalog number BR1921, BR1921a and BR8011). They included 159 IDC, 160 ILC and 50 DCIS. Histopathological subtypes were reviewed by a pathologist (MMW). Clinical annotation included age at diagnosis, tumor size, lymph node status, estrogen receptor, progesterone receptor, human epidermal growth factor (EGF) receptor 2 (HER2). MDA-MB-231, MCF-7, BT-474, and SKBR-3 breast cancer cell lines were from the American Type Culture Collection. JIMT-1 cells were from DSMZ (Germany). Brain metastatic 231-BR cells and HME human mammary epithelial cells (transformed but non-tumorigenic) were a generous gift from Barbara Steeg (Bethesda, USA) and Robert Weinberg (Boston, USA), respectively. Individual cell line authentication was performed after DNA extraction (Promega genomic purification kit, catalog number A1120) and using the GenePrint 10 PCR amplification kit (Promega catalog number B9510). All breast epithelial cell lines were maintained in RPMI-1640 with 10% foetal calf serum (JRH Biosciences) and 2 mM L-glutamine. The neuron-like PC12 cell line was from Ralph A. Bradshaw (University of California San Francisco). They were maintained in Dulbecco's modified eagle medium (DMEM) from Life Technologies (Australia) with 5% foetal calf serum, 10% horse serum (Sigma), and 2 mM L-glutamine. All cell lines were grown in 75 cm^2 tissue culture flasks in a humidified incubator at 37 °C with 5% CO_2. The study was approved by the Human Research Ethic Committee of the University of Newcastle, Australia.

2.2. Immunohistochemistry

After deparaffinization and rehydration of the TMAs following standard procedures, heat induced epitope retrieval was carried out in a citrate based low pH buffer (Vector Laboratories) using a decloaking chamber (Biocare) at 95 °C for 20 min. Immunohistochemistry (IHC) was then performed using an ImmPRESS detection kit (Vector Laboratories) as per the manufacturer's recommendations. Briefly, after inactivation of endogenous peroxidases with H_2O_2, and blocking with 2.5% horse serum, rabbit PGP9.5 antibody (Abcam, catalog number ab15503), or rabbit NGF antibody (Abcam, catalog number ab52918), or non-immune rabbit IgG control (Alpha Diagnostic, catalog number 20009-1-200) were applied at a 1:200 dilution. ImmPRESS HRP anti-rabbit IgG (peroxidase) was then applied to the sections and revealed with DAB peroxidase substrate solution (Vector laboratories). Finally, TMA slides were counterstained with hematoxylin (Gill's formulation, Vector laboratories), dehydrated and cleared in Xylene before mounting in Ultramount #4 mounting media (Thermo Scientific). Imaging was performed using an Axioplan-2 microscope fitted with an AxioCam Mrc5 digital camera (Carl Zeiss AG). The presence or absence of nerve fibers was recorded for

Please cite this article in press as: Pundavela, J., et al., Nerve fibers infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer, Molecular Oncology (2015), http://dx.doi.org/10.1016/j.molonc.2015.05.001

each tumor sample of the TMAs by two independent observers including a pathologist (MMW).

2.3. Digital quantification of immunohistochemistry

For quantification of NGF staining, TMA slides were digitized at 200× absolute resolution using an Aperio AT2 scanner (Leica Biosystems). Quantitative IHC analyses were performed using the Halo™ image analysis platform (Indica Labs) under the supervision of a pathologist. Five random areas containing cancer cells were selected and the pixel intensities of DAB staining calculated using the Area Quantification algorithm. Pixel intensity values were then used to determine h-scores for each core (index ranging from 0 to 300 calculated as the sum of 3 × % of pixels with strong staining + 2 × % of pixels with intermediate staining + 1 × % pixels with weak staining). To compare NGF levels across the cohort, the h-scores were used to divide cases into 4 classifications (0 = h-score <50; 1 = h-score 50–100, 2 = h-score 101–150, 3 = h-score h > 150).

2.4. Association between nerve fibers and clinicopathological parameters

The presence of nerve fibers was compared with clinicopathological parameters (patient age at diagnosis, tumor size, histological subtype, lymph node invasion, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), molecular subtype) and NGF staining intensity. For statistical analysis, simple unadjusted associations between nerve fibers and other pathological variables were performed using a chi-squared test. We used log-linear models to adjust the various bivariate associations for other potential confounders. The log linear models provided a Chi-squared test adjusted for all other variables in the model; these included cancer type (lobular vs ductal), lymph node involvement (yes/no), estrogen receptor positivity (yes/no), progesterone receptor positivity (yes/no), HER2+ (yes/no) and nerve fibers (negative/positive). The model was specified as a Poisson generalized linear model with a log-link function. Using hierarchical nesting of models, all 3-way then 2-way interactions involving nerve fibers were examined. Goodness of fit was tested using G2 Chi-squared statistics, as well as AIC and BIC. These models were fitted using SAS (SAS Institute, North Carolina, USA).

2.5. Preparation of conditioned medium and dot-blot analysis

Subconfluent breast cancer cells were seeded at 5×10^6 cells per 100 mm culture dish and grown in 10 mL serum free media for 24 h. The collected medium was centrifuged (800× *g* for 5 min at 4 °C) and the supernatant was concentrated and desalted using 10-kDa cut-off Amicon Ultra-15 filter unit (Millipore) for 30 min (4000× *g*, 4 °C). The recovered 250 μL concentrate was stored at −80 °C. Dot-blot analysis was performed by spotting 20 μL of concentrated medium onto nitrocellulose membrane using the Bio-Dot microfiltration system (Bio-Rad). Then the membrane was saturated with blocking buffer (LI-COR Biosciences) for 1 h at room temperature before incubation with rabbit anti-NGF polyclonal IgG (Santa Cruz Biotechnology, catalog number sc548) overnight at 4 °C. After washing with PBS containing 0.1% Tween-20, membranes were probed with goat anti-rabbit IR-Dye 680 secondary antisera (LI-COR Biosciences, catalog number 926-68073), then washed twice. Densitometric analysis was performed using the Odyssey infrared imaging system (LI-COR Biosciences).

2.6. Neurite outgrowth assay

The neurotrophic ability of breast cancer cells was tested in co-culture experiments with the neuronal-like PC12 cells and neurite outgrowth was measured. PC12 cells are extensively used for studying neurite elongation (Suter and Miller, 2011). For co-culture experiments, PC12 cells (5×10^4 in 1 ml) were seeded on bottom wells of 12-well Transwell plates (Corning) coated with rat-tail collagen I (Invitrogen). After 24 h, they were serum starved in DMEM containing 1% horse serum. Breast cancer cells were grown in Transwell inserts (12.0 mm in diameter with 0.4 μm pores, Corning). Differentiation of PC12 cells was allowed for 3 days, with or without anti-NGF mouse monoclonal blocking antibody (Alomone, catalog number alm-006) and neurite elongation was measured. PC12 cells exhibiting neurites of at least twice the size of the cell body were considered as differentiated. Pictures were taken using a Zeiss Axiovert 200 inverted microscope fitted with an AxioCam HRm digital camera (Zeiss AG). One-way ANOVA statistical test (GraphPad Prism 5.01) was used.

3. Results

3.1. Breast tumors are infiltrated by nerve fibers

To test the hypothesis that nerve fibers could infiltrate breast tumors, we have explored TMAs containing 319 invasive breast cancers (IDC + ILC) and 50 DCIS by immunohistochemistry against the neuronal marker PGP9.5. Typical morphological features corresponding to both nerves and isolated nerve fibers (axons) were observed (Figure 1). The presence of nerve trunks (composed of many fibers or axons) with perineural invasion (Figure 1A) was occasionally observed in 6 out of the 319 cases of invasive cancers, but this does not constitute a reliable quantification due to the bias introduced by sampling in relation to the location of nerve trunks. Importantly, isolated nerve fibers (axons) were also observed (Figure 1B–H). The reactivity to the neuronal marker PGP9.5 and typical morphology were characteristic of axons. Nerve fibers were localized around cancer cells and adipocytes (Figure 1B), in the tumor stroma next to cancer cells (Figure 1C–F), around arterioles (Figure 1G) and blood vessels (Figure 1H). In Table 1, breast cancers were classified as nerve fiber positive *versus* nerve fiber negative tumors, and comparison was made with clinicopathological parameters. TMAs do not contain enough tissue for analyzing and dissecting precisely nerve fiber densities in tumors and therefore we have chosen to present the data in terms of presence *versus* absence of nerve fibers (this may lead to an underestimation of the innervation). The presence of nerve fibers was detected in 8% of DCIS, 12% of ILC and 28% of IDC ($p = 0.0003$), indicating that

Please cite this article in press as: Pundavela, J., et al., Nerve fibers infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer, Molecular Oncology (2015), http://dx.doi.org/10.1016/j.molonc.2015.05.001

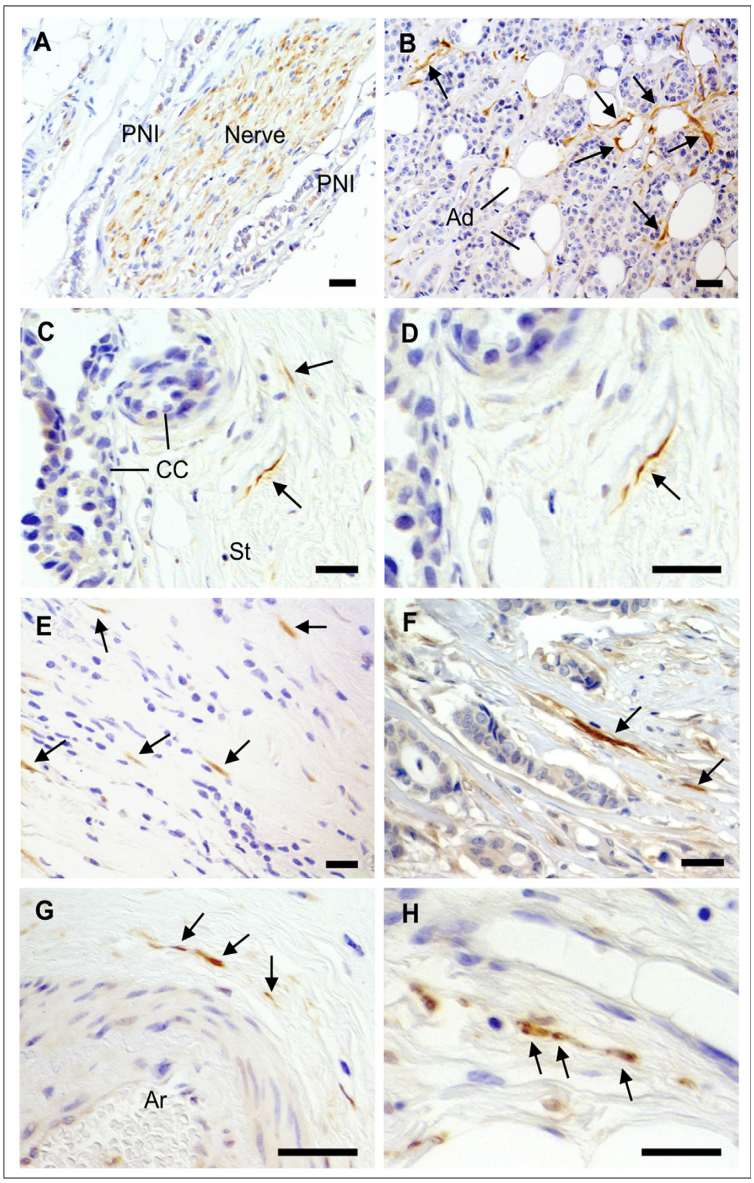


Figure 1 – Detection of nerve fibers in breast cancers. IHC for the neuronal marker PGP9.5 was performed on a series of 319 breast cancer samples. A) Nerve trunks (composed of many nerve fibers), occasionally present in breast tumors, were positive for PGP9.5. Perineural invasion (PNI) could be observed, as shown here. B–H) In some breast cancers, isolated nerve fibers (axons) positive for PGP9.5 were observed and are indicated by arrows. B) Nerve fibers around cancer cells and adipocytes (Ad). C) Nerve fibers in the tumor stroma (St) adjacent to cancer cells (CC). D) Enlargement of C. E, F) Nerve fibers among scattered breast cancer cells and in tumor stroma. G) Nerve fibers around an arteriole (Ar). H) Nerve fibers close to a thin walled blood vessel in the tumor stroma. Scale bar = 50 μm.

nerve fibers are predominantly associated with invasive ductal carcinomas. This association was confirmed in Log-Linear modeling, two-way analysis ($p < 0.001$). In invasive breast tumors, there was no association between the presence of nerve fibers and age at diagnosis, HER2, estrogen receptor, progesterone receptor, tumor size and molecular subtype (defined as TNBC: ER–/PR–/HER2–, luminal A: ER+ and/or PR+/HER2–, luminal B: ER+ and/or PR+/HER2+, HER2+: ER–/PR–/HER2+). The tumors that presented innervation were not enriched in any particular molecular subtypes. A more detailed analysis of innervation in function of ER/PR/HER2 status is shown in Supplementary Table 1 and indicates the absence of statistically significant differences between the subgroups.

Please cite this article in press as: Pundavela, J., et al., Nerve fibers infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer, Molecular Oncology (2015), http://dx.doi.org/10.1016/j.molonc.2015.05.001

Table 1 – Association between the presence of nerve fibers and clinicopathological parameters in breast carcinomas.

Parameter	Nerve fibers negative	Nerve fibers positive	*p*-value
All cases (*n* = 369)	301 (82%)	68 (18%)	
Pathological subtype			**0.0003**[a]
DCIS (n = 50)	46 (92%)	4 (8%)	
ILC (n = 160)	140 (88%)	20 (12%)	
IDC (n = 159)	115 (72%)	44 (28%)	
Clinical parameters in invasive carcinomas			
Patient age			
50≤ (n = 183)	142 (77%)	41 (22%)	0.1201
>50 (n = 136)	115 (84%)	21 (15%)	
Lymph node status (N)			**0.0031**[a]
Negative (n = 164)	140 (85%)	24 (15%)	
Positive (n = 135)	97 (72%)	38 (28%)	
Undetermined (n = 20)	16 (80%)	4 (20%)	
HER2			0.2162
HER2 negative (n = 252)	205 (81%)	47 (19%)	
HER2 positive (n = 67)	50 (75%)	17 (15%)	
Estrogen receptor			0.3093
ER negative (n = 182)	149 (82%)	33 (18%)	
ER positive (n = 137)	106 (77%)	31 (13%)	
Progesterone receptor			0.8143
PR negative (n = 208)	167 (80%)	41 (20%)	
PR positive (n = 111)	88 (79%)	23 (21%)	
Molecular subtype			0.5801
TNBC (n = 123)	98 (80%)	25 (20%)	
Luminal A (n = 129)	107 (83%)	22 (17%)	
Luminal B (n = 34)	25 (74%)	9 (26%)	
HER2+ (n = 33)	25 (76%)	8 (24%)	
Tumor size (T)			0.5284
1 (n = 25)	19 (76%)	6 (14%)	
2 (n = 228)	183 (80%)	45 (20%)	
3 (n = 32)	28 (88%)	4 (12%)	
4 (n = 30)	22 (73%)	8 (27%)	
Nerve growth factor			**0.0001**[a]
NGF negative (n = 167)	149 (89%)	18 (11%)	
NGF positive (n = 152)	107 (70%)	45 (30%)	

DCIS = ductal carcinomas in situ; ER = estrogen receptor; HER2 = Human EGF receptor 2; IDC = invasive ductal carcinomas; ILC = invasive lobular carcinomas; NGF = nerve growth factor; TNBC = triple negative breast cancer.
a Statistically significant *p*-values ($p < 0.05$ using chi-square test). Molecular subtypes were defined as TNBC: ER−/PR−/HER2−, luminal A: ER+ and/or PR+/HER2−, luminal B: ER+ and/or PR+/HER2+, HER2+: ER−/PR−/HER2+.

3.2. *The presence of nerve fibers in invasive breast tumors is associated with lymph node invasion and NGF production*

The presence of nerve fibers was associated with lymph node invasion and NGF production in cancer cells (Table 1). Individual nerve fibers were observed in only 15% of invasive tumors with no lymph node invasion whereas 28% of lymph node positive tumors contained nerve fibers (p = 0.0031), indicating a relationship between the presence of nerve fibers and metastatic potential/poorer prognosis. The association between nerve fibers and lymph node invasion was confirmed in Log-linear modeling (p = 0.0064 in two-way analysis). Together this association with lymph node invasion and the fact that only 8% of DCIS presented with nerve fibers indicate that the presence of nerve fibers in breast tumors is related to aggressiveness/invasiveness.

In addition, as we have previously shown that the neurotrophic growth factor NGF is expressed in breast tumors (Adriaenssens et al., 2008), we tested the hypothesis that the presence of nerve fibers in breast cancers could be related to NGF expression. Interestingly, there was an association between the presence of nerve fibers and the expression of NGF in cancer cells (Table 1). Nerve fibers were observed in only 11% of NGF negative tumors (h-score<50), as compared to 30% of NGF positive tumors (h-score≥50) (p = 0.0001). The digital quantification of NGF intensity staining is presented (Figure 2). NGF intensity staining (h-score) was significantly higher in IDC than in DCIS and ILC ($p < 0.0001$) (Figure 2A), thus corroborating the higher proportion of IDC presenting with nerve fibers. The frequency distribution of NGF staining intensity in DCIS, ILC and IDC is presented as categories (Figure 2B), with 0 = h-score <50, 1 = h-score 50–100, 2 = h-score 101–150, 3 = h-score>150. The percentage of cases with NGF labeling was 2% in DCIS, 15% in ILC and 79% in IDC ($p < 0.0001$). In invasive tumors (Figure 2C), the proportion of cases with high NGF labeling (intensity labeling 2 and 3) was 12% in nerve fibers negative tumors and 31% in nerve fibers positive tumors ($p < 0.0001$), confirming the association between NGF and the presence of nerve fibers. Overall, the Spearman correlation factor between nerve fibers and NGF level was 0.28 ($p < 0.0001$). Furthermore, in serial sections with NGF and PGP9.5 staining, nerve fibers were observed around cancer cells producing NGF (Figure 3). Together, these results suggested that NGF produced by breast cancer cells could participate in tumor infiltration by nerves and have prompted an examination of a NGF-mediated neurotrophic effect (ability to induce neuronal outgrowth) of breast cancer cells.

3.3. *Breast cancer cells can induce neuronal outgrowth through NGF secretion*

Given the association between the presence of nerve fibers and the production of NGF in breast tumors, the neurotrophic ability of breast cancer cells was investigated. Co-cultures between breast cancer cell lines and the neuronal-like PC12 cells (Figure 4A) were performed and neurite outgrowth of PC12 cells was measured as a percentage of cells with elongated neurites (Figure 4B). MCF-7, MDA-MB-231 and 231-BR induced a strong neurite outgrowth, whereas only a slight induction was observed with SKBR-3 and JIMT-1. In contrast, no neurite outgrowth was detected with BT-474. Interestingly, the non-tumorigenic HME cells were not able to induce neurite outgrowth. Representative pictures of PC12 cells co-cultured in presence of breast cancer cells are shown (Figure 4C). NGF was quantified in dot blot analyses of conditioned media from breast cancer cell lines (Figure 4D). The dot blot was prepared from an equivalent number of cells and the densitometric quantification indicated different levels of NGF between cell lines. Interestingly, the levels of NGF in the conditioned media of breast cancer cells partly corroborated their ability to induce neurite outgrowth (as shown in Figure 4B), suggesting that NGF participates in the

Please cite this article in press as: Pundavela, J., et al., Nerve fibers infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer, Molecular Oncology (2015), http://dx.doi.org/10.1016/j.molonc.2015.05.001

neurotrophic effect induced by breast cancer cells. MCF-7 exhibited the highest level of NGF secretion and the highest neurotrophic effect (as reported in panel B). The non-tumorigenic HME had a low level of NGF and exhibited a limited neurotrophic effect. However, MDA-MB-231 cells exhibited a significant neurotrophic effect whereas the level of NGF was low, and BT-474 had an intermediate NGF level and only a low impact on neuritogenesis. This suggested that NGF is probably not the only neurotrophic factor produced by breast cancer cells and able to stimulate neuron outgrowth. The involvement of NGF in breast cancer-induced neurite outgrowth was confirmed by use of blocking antibodies against NGF (Figure 4E). As shown with MCF-7 cells, blocking antibodies against NGF could partly inhibit breast cancer cell-induced neurite outgrowth whereas a control IgG antibody had no effect. Representative pictures showing the effect of anti-NGF antibody on neurite outgrowth induced by MCF-7 cells are shown (Figure 4F). The inhibitory activity of anti-NGF blocking antibody for breast cancer-induced neurite outgrowth has been confirmed using the 50B11 cell line (Supplementary Figure 1), which is derived from dorsal root ganglia and morphologically responsive to NGF (Bhattacherjee et al., 2014). Together these data indicate that breast cancer cells have the ability to stimulate axonogenesis through the production and release of NGF, and that other neurotrophic factors may also be involved.

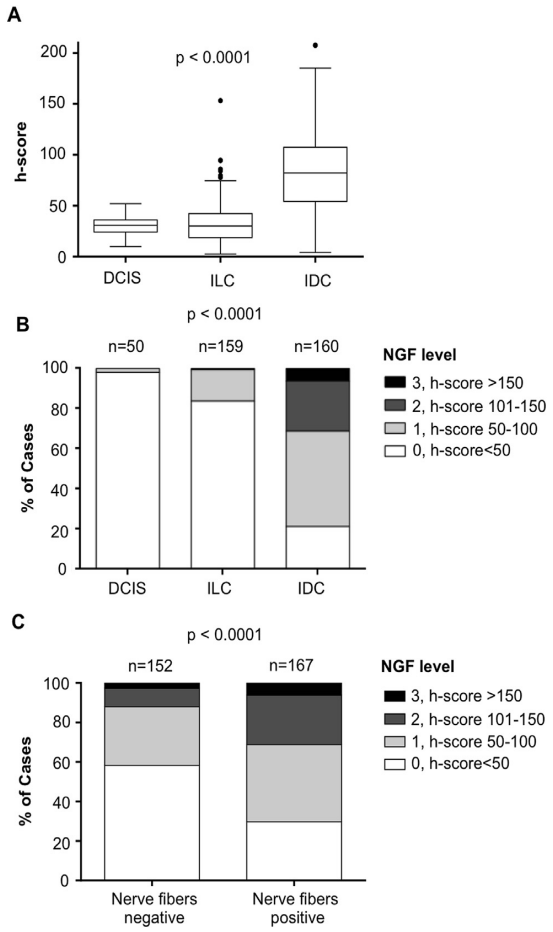


Figure 2 – Frequency distribution of NGF level in breast cancers according to the presence of nerve fibers. NGF levels were obtained after digital quantification. A) Distribution of NGF intensity staining (h-score) in ductal carcinomas in situ (DCIS), invasive lobular carcinomas (ILC) and invasive ductal carcinomas (IDC). Box and Whisker plots comparing median NGF levels using h-scores as a measure of IHC staining (n = 50, 160 and 160, respectively). The box limits indicate the 25th and 75th percentiles with the whiskers extending 1.5 times the interquartile range from the 25th and 75th percentiles (outliers are represented by dots; prepared using BoxPlotR). B) Distribution of NGF staining intensity in DCIS, ILC and IDC. Categorization is presented as 0 = h-score < 50, 1 = h-score 50–100, 2 = h-score 101–150, 2 = h-score > 150. C) Distribution of NGF staining intensity in invasive tumors (nerve fibers positive *versus* nerve fibers negative tumors). Categories of NGF staining (0, 1, 2, 3) were the same as in B. Tumors presenting with nerve fibers were more likely to have higher NGF expression than tumors without nerve fibers. Number of cases (n) is indicated. ***One-way ANOVA was used for A and Chi square for B and D.

4. Discussion

The tumor microenvironment is crucial to breast cancer progression and the interaction of breast cancer cells with the components of the stroma, endothelial and immune cells, fibroblasts and extracellular matrix, is well established (Hanahan and Weinberg, 2011). In contrast, the presence of nerve fibers in the breast tumor microenvironment and a possible interaction with cancer cells has not been studied in detail. The present study demonstrates the presence of thin and isolated nerve fibers (axons) in the breast tumor microenvironment and their association with NGF production in cancer cells. In prostate cancer, nerve infiltration correlates with tumor aggressiveness (Magnon et al., 2013), and is driven by the production of proNGF (Pundavela et al., 2014) and granulocyte colony-stimulating factor (G-CSF) (Dobrenis et al., 2015). The results presented here suggest that a similar situation occurs in breast cancer as infiltration by nerve fibers was found associated to lymph node invasion. Lymph node status is the single most important prognostic variable for the management of patients with primary breast cancer. However, the occurrence of false negatives, along with heterogeneity of clinical outcomes among lymph node positive patients, highlights the need to improve the classification and management of invasive breast cancer. This study points to the potential value of using the presence of nerve fibers as a new predictive biomarker in breast cancer.

The production and release of neurotrophic factors by breast cancer cells has been described (Hondermarck, 2012). NGF expression is increased in breast cancer cells as compared to normal breast epithelial cells (Adriaenssens et al., 2008), resulting in an autocrine stimulation of breast cancer cells through the tyrosine kinase receptor TrkA and the TNF-receptor family member $p75^{NTR}$ (Lagadec et al., 2009; Verbeke et al., 2010). However, until now, the possibility that NGF produced by breast cancer cells could stimulate tumor nerve infiltration had not been investigated. The present

Please cite this article in press as: Pundavela, J., et al., Nerve fibers infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer, Molecular Oncology (2015), http://dx.doi.org/10.1016/j.molonc.2015.05.001

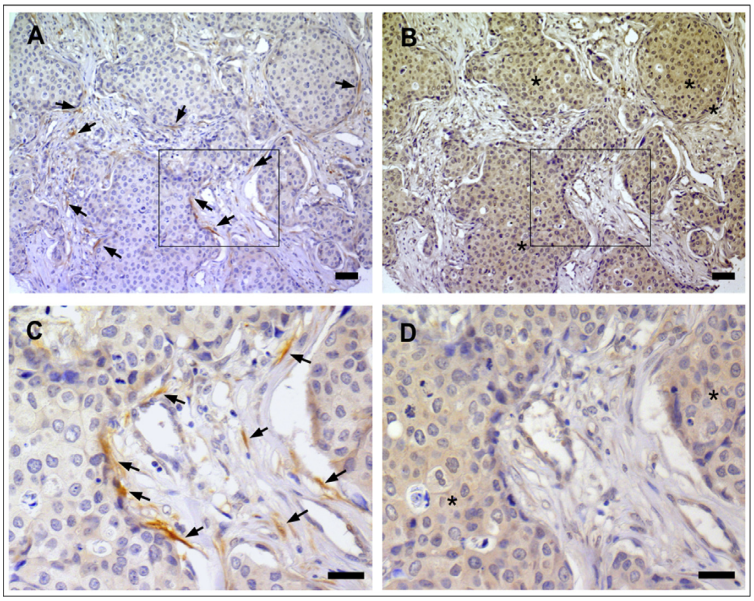


Figure 3 – Co-localization between nerve fibers and NGF in breast cancer. A) IHC for PGP9.5 indicating the presence of many nerve fibers in the stroma and along cancer cells of an invasive ductal carcinoma. Arrows point to few nerve fibers. B) IHC against NGF in a section serial to that presented in panel A. NGF immunoreactivity (indicated by stars) was observed in cancer cells adjacent to nerve fibers. C) Enlargement of the area boxed in panel A. D) Enlargement of the area boxed in panel B. Scale bar = 50 μm.

study reveals that breast cancer cells can activate neuronal outgrowth through a NGF-mediated mechanism. In bone metastases of breast cancer, one study has already shown that NGF production by tumor cells can attract nerves (Bloom et al., 2011) and our study demonstrates that nerve infiltration is also a characteristic of primary breast tumors that is partially driven by NGF production. However, the correlation between NGF and nerve outgrowth, both in tumor samples and in cell cultures, was only partial, suggesting that NGF is not the only factor involved. There are many growth factors (neurotrophins and others) that can exhibit a neurotrophic activity, and several are produced in breast cancers. Brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (Hernandez-Bedolla et al., 2015; Vanhecke et al., 2011), Artemin (Kang et al., 2009) or fibroblast growth factors (Penault-Llorca et al., 1995), as well as axon guidance molecules such as netrins (Harburg and Hinck, 2011) are expressed by breast cancer cells and could also contribute to the attraction of nerve fibers. The development of the nervous system involves a variety of neurotrophic molecules that act on different neuronal subtypes, and it is possible that a similar diversity of mechanisms also participates in tumor innervation. Further investigations are warranted to clarify the possible involvement of neurotrophic factors and axon guidance molecules in the infiltration of breast tumors by nerve fibers.

The presence of nerve fibers could be particularly relevant in terms of tumor growth and metastasis, via the secretion of active neuropeptides and neurotransmitters. Breast cancer cells have been reported to express receptors for a number of neuropeptides and neurotransmitters, like norepinephrine and epinephrine (Luthy et al., 2009) or substance P (Garcia-Recio et al., 2013). As a consequence tumor cells are able to transduce neurotransmitter-induced intracellular signaling pathways, which have been described to eventually lead to the activation of their growth and metastasis (Entschladen et al., 2004). The presence of nerve fibers in the breast tumor microenvironment suggests that they could liberate neurotransmitters directly in the vicinity of breast cancer cells. We have observed tyrosine hydroxylase positivity in some nerve trunks as well as axons (Supplementary Figure 2), indicating that some of the nerve fibers present in breast cancer are of sympathetic origin and could therefore liberate catecholamines. It has been shown that the expression of the beta-adrenergic receptor for catecholamines is associated with a poor clinical outcome in breast cancer patients (Powe et al., 2011) and that stress-induced activation of the sympathetic nervous system can induce a metastatic switch (Sloan et al., 2010). The mechanism involved the stimulation of beta-adrenergic receptors at the surface of breast cancer cells by circulating catecholamines, but our study suggests that a local production by nerves may also be involved. In addition, epidemiological studies have suggested that blockers of the beta-adrenergic receptors, traditionally used for the treatment of cardiovascular disorders and anxiety, can increase breast cancer patient survival (Barron et al., 2011; Melhem-Bertrandt et al., 2011); the mechanism is unclear, but beta-blockers could potentially inhibit the stimulatory effect of catecholamines liberated by nerves. Although further experiments are necessary to test these hypotheses and determine if infiltrating nerve fibers have an impact on cancer and

Please cite this article in press as: Pundavela, J., et al., Nerve fibers infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer, Molecular Oncology (2015), http://dx.doi.org/10.1016/j.molonc.2015.05.001

ARTICLE IN PRESS

8 MOLECULAR ONCOLOGY XXX (2015) 1–10

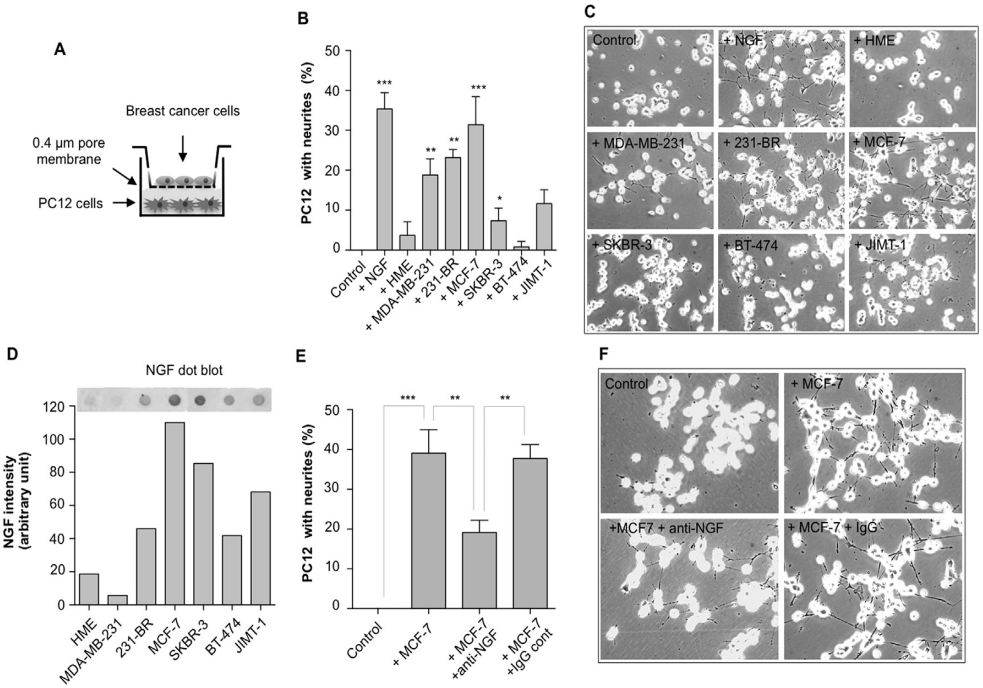


Figure 4 – NGF-mediated neurotrophic effect of breast cancer cells. A) Co-culture experiments were performed in Transwell Boyden chambers with breast epithelial cells in the upper part and PC12 cells in the lower part. B) Some breast cancer cell lines were able to induce a neurotrophic effect on PC12 cells. Neurite outgrowth was induced in presence of MDA-MB-231, 231-BR, MCF-7, SKBR-3, JIMT-1, but not in presence of BT-474 and the non-tumorigenic HME. A negative control (with no breast cancer cells) and a positive control (addition of 50 ng/ml NGF) have been added. The results represent the mean of 3 independent experiments ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 for comparison with control. C) Representative pictures showing the effect of breast cancer cell lines and HME on PC12 cells. Quantifications are presented in panel B. D) Dot-blot analysis for the detection of NGF in breast cancer cell conditioned media and quantification of NGF signal intensity in different breast epithelial cells. E) Impact of blocking anti-NGF antibodies on breast cancer cell-induced neurite outgrowth. MCF-7 cells were co-cultured for 72 h with PC12 cells in presence or absence of anti-NGF blocking antibodies (1 µg/ml). Control was without MCF-7 cells, and isotype antibodies were also tested. The results represent the mean of 3 independent experiments ± SD. ***p < 0.001. F) Representative pictures corresponding to the experiment described in panel E.

stromal cells, our results open the theoretical possibility that, similarly to prostate cancer (Magnon et al., 2013), nerve fibers could be involved in breast cancer progression.

In conclusion, the nerve-breast cancer connection described here bridges a gap in knowledge about the neuronal component of the breast tumor microenvironment and points to NGF as a driver of nerve infiltration. This opens a new perspective of crosstalk between nerves and breast cancer cells, and warrants more studies to investigate the impact of nerve fibers in breast cancer progression.

Contributions

SR performed the immunohistochemistry. Tissue slide analyses were performed by MMW (histopathologist), and confirmed by PJ (neuroanatomist) and HH. Digital quantification of IHC was performed by RT. JP performed all *in vitro* experiments and participated in IHC; he also prepared all Figures and Tables. JA supervised the statistical analyses. SF contributed to cell culture. HH and PJ conceived the study. HH, MMW, RJS, RAB and JFF supervised the study. HH drafted the manuscript. All authors have read and approved the final manuscript.

Conflict of interest

The authors disclosed no competing interests.

Acknowledgments

This work was supported by the University of Newcastle, Australia and the Hunter Cancer Research Alliance. We thank Dr. Christopher Oldmeadow from the Clinical Research Design, IT, and Statistical Support (CReDITSS) Unit of the University of Newcastle.

Please cite this article in press as: Pundavela, J., et al., Nerve fibers infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer, Molecular Oncology (2015), http://dx.doi.org/10.1016/j.molonc.2015.05.001

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2015.05.001.

REFERENCES

Adriaenssens, E., Vanhecke, E., Saule, P., Mougel, A., Page, A., Romon, R., Nurcombe, V., Le Bourhis, X., Hondermarck, H., 2008. Nerve growth factor is a potential therapeutic target in breast cancer. Cancer Res. 68, 346–351.

Bapat, A.A., Hostetter, G., Von Hoff, D.D., Han, H., 2011. Perineural invasion and associated pain in pancreatic cancer. Nat. Rev. Cancer 11, 695–707.

Barron, T.I., Connolly, R.M., Sharp, L., Bennett, K., Visvanathan, K., 2011. Beta blockers and breast cancer mortality: a population- based study. J. Clin. Oncol. 29, 2635–2644.

Bhattacherjee, A., Liao, Z., Smith, P.G., 2014. Trophic factor and hormonal regulation of neurite outgrowth in sensory neuron-like 50B11 cells. Neurosci. Lett. 558, 120–125.

Bloom, A.P., Jimenez-Andrade, J.M., Taylor, R.N., Castaneda-Corral, G., Kaczmarska, M.J., Freeman, K.T., Coughlin, K.A., Ghilardi, J.R., Kuskowski, M.A., Mantyh, P.W., 2011. Breast cancer-induced bone remodeling, skeletal pain, and sprouting of sensory nerve fibers. J. Pain 12, 698–711.

Campbell, J.P., Karolak, M.R., Ma, Y., Perrien, D.S., Masood-Campbell, S.K., Penner, N.L., Munoz, S.A., Zijlstra, A., Yang, X., Sterling, J.A., Elefteriou, F., 2012. Stimulation of host bone marrow stromal cells by sympathetic nerves promotes breast cancer bone metastasis in mice. PLoS Biol. 10, e1001363.

Dobrenis, K., Gauthier, L.R., Barroca, V., Magnon, C., 2015. Granulocyte colony-stimulating factor off-target effect on nerve outgrowth promotes prostate cancer development. Int. J. Cancer 136, 982–988.

Entschladen, F., Drell, T.L.t, Lang, K., Joseph, J., Zaenker, K.S., 2004. Tumour-cell migration, invasion, and metastasis: navigation by neurotransmitters. Lancet Oncol. 5, 254–258.

Garcia-Recio, S., Fuster, G., Fernandez-Nogueira, P., Pastor-Arroyo, E.M., Park, S.Y., Mayordomo, C., Ametller, E., Mancino, M., Gonzalez-Farre, X., Russnes, H.G., Engel, P., Costamagna, D., Fernandez, P.L., Gascon, P., Almendro, V., 2013. Substance P autocrine signaling contributes to persistent HER2 activation that drives malignant progression and drug resistance in breast cancer. Cancer Res. 73, 6424–6434.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646–674.

Harburg, G.C., Hinck, L., 2011. Navigating breast cancer: axon guidance molecules as breast cancer tumor suppressors and oncogenes. J. Mammary Gland Biol. Neoplasia 16, 257–270.

Hernandez-Bedolla, M.A., Carretero-Ortega, J., Valadez-Sanchez, M., Vazquez-Prado, J., Reyes-Cruz, G., 2015. Chemotactic and proangiogenic role of calcium sensing receptor is linked to secretion of multiple cytokines and growth factors in breast cancer MDA-MB-231 cells. Biochim. Biophys. Acta 1853, 166–182.

Hondermarck, H., 2012. Neurotrophins and their receptors in breast cancer. Cytokine Growth Factor Rev. 23, 357–365.

Huang, D., Su, S., Cui, X., Shen, X., Zeng, Y., Wu, W., Chen, J., Chen, F., He, C., Liu, J., Huang, W., Liu, Q., Su, F., Song, E., Ouyang, N., 2014. Nerve fibers in breast cancer tissues indicate aggressive tumour progression. Medicine (Baltimore) 93, e172.

Jobling, P., Pundavela, J., Oliverira, S.M.R., Roselli, S., Walker, M.M., Hondermarck, H., 2015. Nerve-cancer cell crosstalk: a novel promoter of tumour progression. Cancer Res. 75, 1–5.

Kang, J., Perry, J.K., Pandey, V., Fielder, G.C., Mei, B., Qian, P.X., Wu, Z.S., Zhu, T., Liu, D.X., Lobie, P.E., 2009. Artemin is oncogenic for human mammary carcinoma cells. Oncogene 28, 2034–2045.

Lagadec, C., Meignan, S., Adriaenssens, E., Foveau, B., Vanhecke, E., Romon, R., Toillon, R.A., Oxombre, B., Hondermarck, H., Le Bourhis, X., 2009. TrkA overexpression enhances growth and metastasis of breast cancer cells. Oncogene 28, 1960–1970.

Luthy, I.A., Bruzzone, A., Pinero, C.P., Castillo, L.F., Chiesa, I.J., Vazquez, S.M., Sarappa, M.G., 2009. Adrenoceptors: non conventional target for breast cancer? Curr. Med. Chem. 16, 1850–1862.

Magnon, C., Hall, S.J., Lin, J., Xue, X., Gerber, L., Freedland, S.J., Frenette, P.S., 2013. Autonomic nerve development contributes to prostate cancer progression. Science 341, 1236361.

Marchesi, F., Piemonti, L., Mantovani, A., Allavena, P., 2010. Molecular mechanisms of perineural invasion, a forgotten pathway of dissemination and metastasis. Cytokine Growth Factor Rev. 21, 77–82.

Melhem-Bertrandt, A., Chavez-Macgregor, M., Lei, X., Brown, E.N., Lee, R.T., Meric-Bernstam, F., Sood, A.K., Conzen, S.D., Hortobagyi, G.N., Gonzalez-Angulo, A.M., 2011. Beta-blocker use is associated with improved relapse-free survival in patients with triple-negative breast cancer. J. Clin. Oncol. 29, 2645–2652.

Ondicova, K., Mravec, B., 2010. Role of nervous system in cancer aetiopathogenesis. Lancet Oncol. 11, 596–601.

Penault-Llorca, F., Bertucci, F., Adelaide, J., Parc, P., Coulier, F., Jacquemier, J., Birnbaum, D., deLapeyriere, O., 1995. Expression of FGF and FGF receptor genes in human breast cancer. Int. J. Cancer 61, 170–176.

Powe, D.G., Voss, M.J., Habashy, H.O., Zanker, K.S., Green, A.R., Ellis, I.O., Entschladen, F., 2011. Alpha- and beta-adrenergic receptor (AR) protein expression is associated with poor clinical outcome in breast cancer: an immunohistochemical study. Breast Cancer Res. Treat. 130, 457–463.

Pundavela, J., Demont, Y., Jobling, P., Lincz, L.F., Roselli, S., Thorne, R., Bond, D., Bradshaw, R.A., Walker, M.M., Hondermarck, H., 2014. ProNGF correlates with Gleason score and is a potential driver of nerve infiltration in prostate cancer. Am. J. Pathol. 184, 3156–3162.

Sarhadi, N.S., Shaw Dunn, J., Lee, F.D., Soutar, D.S., 1996. An anatomical study of the nerve supply of the breast, including the nipple and areola. Br. J. Plast. Surg. 49, 156–164.

Skaper, S.D., 2012. The neurotrophin family of neurotrophic factors: an overview. Methods Mol. Biol. 846, 1–12.

Sloan, E.K., Priceman, S.J., Cox, B.F., Yu, S., Pimentel, M.A., Tangkanangnukul, V., Arevalo, J.M., Morizono, K., Karanikolas, B.D., Wu, L., Sood, A.K., Cole, S.W., 2010. The sympathetic nervous system induces a metastatic switch in primary breast cancer. Cancer Res. 70, 7042–7052.

Suter, D.M., Miller, K.E., 2011. The emerging role of forces in axonal elongation. Prog. Neurobiol. 94, 91–101.

Vanhecke, E., Adriaenssens, E., Verbeke, S., Meignan, S., Germain, E., Berteaux, N., Nurcombe, V., Le Bourhis, X., Hondermarck, H., 2011. Brain-derived neurotrophic factor and neurotrophin-4/5 are expressed in breast cancer and can be targeted to inhibit tumor cell survival. Clin. Cancer Res. 17, 1741–1752.

Verbeke, S., Meignan, S., Lagadec, C., Germain, E., Hondermarck, H., Adriaenssens, E., Le Bourhis, X., 2010. Overexpression of p75(NTR) increases survival of breast cancer cells through p21(waf1). Cell Signal. 22, 1864–1873.

Please cite this article in press as: Pundavela, J., et al., Nerve fibers infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer, Molecular Oncology (2015), http://dx.doi.org/10.1016/j.molonc.2015.05.001

Zhao, C.M., Hayakawa, Y., Kodama, Y., Muthupalani, S., Westphalen, C.B., Andersen, G.T., Flatberg, A., Johannessen, H., Friedman, R.A., Renz, B.W., Sandvik, A.K., Beisvag, V., Tomita, H., Hara, A., Quante, M., Li, Z., Gershon, M.D., Kaneko, K., Fox, J.G., Wang, T.C., Chen, D., 2014a. Denervation suppresses gastric tumorigenesis. Sci. Transl Med. 6, 250ra115.

Zhao, Q., Yang, Y., Liang, X., Du, G., Liu, L., Lu, L., Dong, J., Han, H., Zhang, G., 2014b. The clinicopathological significance of neurogenesis in breast cancer. BMC Cancer 14, 484.

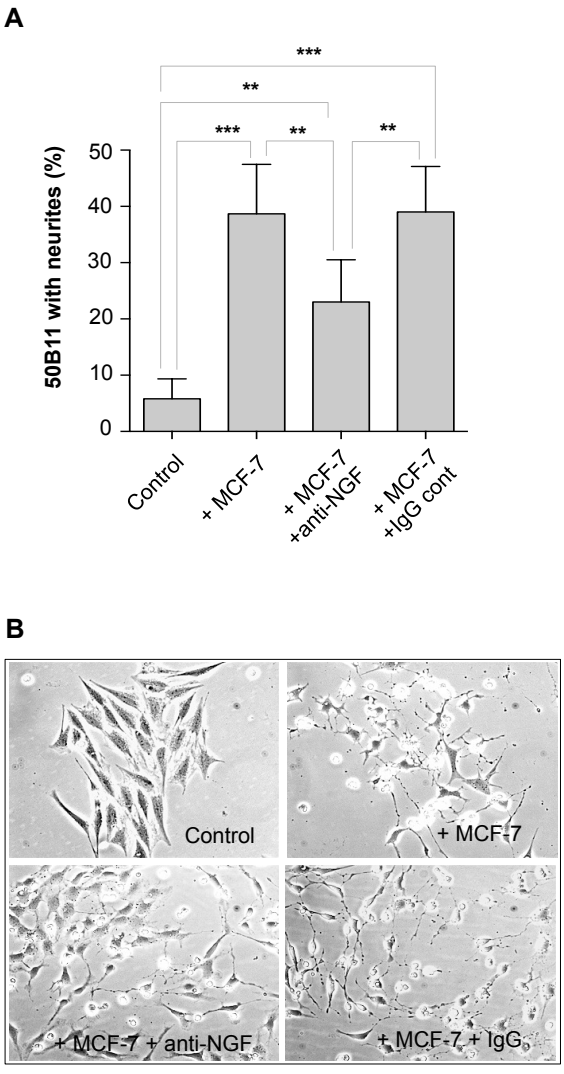
Please cite this article in press as: Pundavela, J., et al., Nerve fibers infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer, Molecular Oncology (2015), http://dx.doi.org/10.1016/j.molonc.2015.05.001

4.3 SUPPLEMENTAL FILES

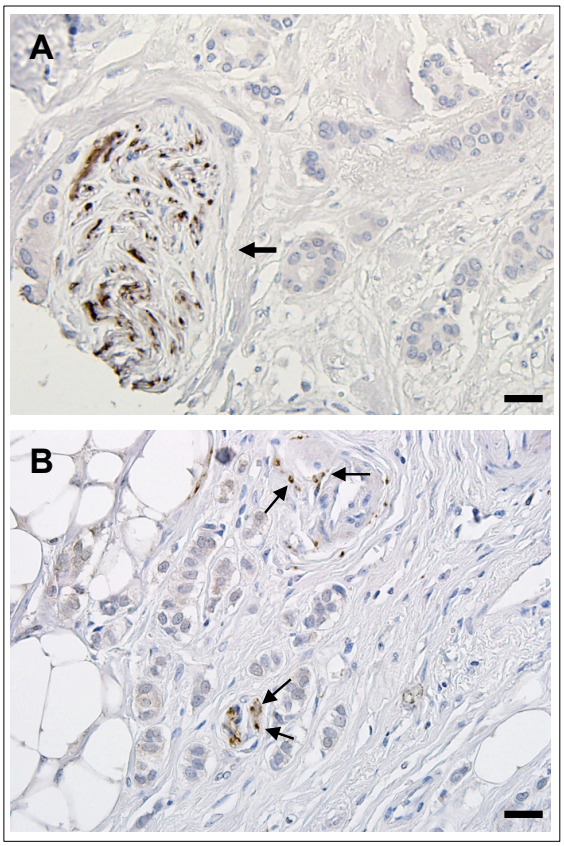
Supplementary Figure Legends

Supplementary Figure 1. NGF-mediated neurotrophic effect of breast cancer cells for 50B11 cells from dorsal root ganglia. Immortalized dorsal root ganglia (DRG) neurons 50B11 were obtained from Dr A. Höke (John Hopkins University, Baltimore USA). The co-culture with breast cancer cells was performed as described for PC12 cells in the Material and Methods section. For co-culture with the breast cancer cells, the same protocol as for PC12 cells was used (see Material and Methods section), but the culture media included 5μM forskolin (necessary to obtain neurite outgrowth with these cells). **A)** MCF-7 cells were able to induce neurite outgrowth in 50B11 cells. This neurotrophic effect was partially inhibited by addition of anti-NGF antibody but not by IgG control. B) Representative pictures of each experimental condition are shown. The results represent the mean of 3 independent experiments +/- SD. *** $p<0.001$; ** $p<0.05$.

Supplementary Figure 2. Detection of tyrosine hydroxylase positive nerve fibers in breast cancer. Tyrosine hydroxylase was detected by IHC using the same protocol as described in Material and Methods, with anti-tyrosine hydroxylase (Millipore, catalogue number AB152). **A)** Nerve trunk composed of many fibers positive for tyrosine hydroxylase is shown by an arrow. **B)** Individual nerve fibers (axons) positive for tyrosine hydroxylase are indicated by arrows. Scale bar=50 μ m.



Supplementary Fig. 1



Supplementary Fig. 2

CHAPTER 5 NERVE-CANCER CELL CROSSTALK: A NOVEL PROMOTER OF TUMOR PROGRESSION

5.1 PREFACE

Contained in this chapter is the manuscript entitled "***Nerve-cancer cell crosstalk: a novel promoter of tumor progression***" accepted for publication in the journal *Cancer Research*. It serves as the discussion segment of this thesis, which places into perspective the evidences reported in Chapter 2 and Chapter 3. There are two important concepts described in this chapter that builds into the argument of the thesis. Primarily, shown under the heading "*Nerves in the tumor microenvironment and their impact on cancer progression*", elaborated the role of the nervous system in cancer progression. It is described that nerves infiltrating tumors release neurotransmitters and neuropeptides that activate cancer progression via neurotransmitter receptors present on the surface membranes of cancer cells. Secondly, tumors exhibit the ability to stimulate neuronal growth through the release of neurotrophic growth factors. This is discussed under the heading "*Neurogenic activity of tumor cells: the role of neurotrophic growth factors*". A commentary in regards to the therapeutic potentials and clinical relevance is discussed under the heading "*Potential anti-neurogenic therapies in cancer*". In order to further contend the imperative significance on the role of nerve infiltration in cancer biology, the section of "*Future directions*" will explain further strategies in the field.

5.2 PUBLICATION

Published OnlineFirst March 20, 2015; DOI: 10.1158/0008-5472.CAN-14-3180

Review

Cancer Research

Nerve–Cancer Cell Cross-talk: A Novel Promoter of Tumor Progression

Phillip Jobling[1,2], Jay Pundavela[1,2], Sonia M.R. Oliveira[1,2], Séverine Roselli[1,2], Marjorie M. Walker[2,3], and Hubert Hondermarck[1,2]

Abstract

Recent studies have revealed the essential role played by nerves in tumor progression. Nerves have been shown to infiltrate the tumor microenvironment and actively stimulate cancer cell growth and dissemination. This mechanism involves the release of neurotransmitters, such as catecholamines and acetylcholine, directly into the vicinity of cancer and stromal cells to activate corresponding membrane receptors. Conversely, the secretion of neurotrophic growth factors by cancer cells drives the outgrowth of nerves in solid tumors. This reciprocal interaction between nerves and cancer cells provides new insights into the cellular and molecular bases of tumorigenesis and points to the potential utility of antineurogenic therapies. This review will discuss our evolving understanding of the cross-talk between nerves and cancer cells. *Cancer Res; 75(8); 1–5. ©2015 AACR.*

Introduction

The peripheral nervous system can be viewed as a neuronal circuit that connects all body parts and organs to the central nervous system and thus the brain. Sensory and motor nerves are mediators of environmental adaptation to the outside world through cognitive integration and muscular movement. Interestingly, the same concept applies to internal organs via autonomic nerves. Sympathetic and parasympathetic nerves reach most internal organs and orchestrate tissue homeostasis through direct innervation and release of neurotransmitters such as catecholamines and acetylcholine. Despite early studies dating back from the 1940s and showing an impact of denervation in cancer (1–3), the role of nerves in cancer initiation and progression has remained unclear. However, recent evidence has led to the crystallization of a new paradigm: that the infiltration of the tumor microenvironment by nerves, termed tumor neoneurogenesis or axonogenesis, plays an active role in cancer progression. Infiltrating nerve fibers stimulate tumor growth and dissemination, and reciprocally tumor cells drive nerve outgrowth in a cross-talk that contributes to tumor progression. This review aims to outline the latest developments about the nerve–cancer cell interaction and to explore the potential value of antineurogenic therapies.

[1]School of Biomedical Sciences and Pharmacy, University of Newcastle, Callaghan New South Wales, Australia. [2]Hunter Medical Research Institute, University of Newcastle, Callaghan New South Wales, Australia. [3]School of Medicine and Public Health, Faculty of Health and Medicine, University of Newcastle, Callaghan New South Wales, Australia.

Corresponding Author: Hubert Hondermarck, School of Biomedical Sciences and Pharmacy, Life Science Building, University of Newcastle, University Drive, Callaghan NSW 2308, Australia. Phone: 61-492-18830; Fax: 61-2492-18831; E-mail: hubert.hondermarck@newcastle.edu.au

doi: 10.1158/0008-5472.CAN-14-3180

Nerves in the Tumor Microenvironment and Their Impact on Cancer Progression

The tumor microenvironment is crucial to cancer progression. The interaction of cancer cells with the stroma, including endothelial cells, immune cells, fibroblasts, and the extracellular matrix, has been established, and this has led to innovative anticancer therapies, for example, those targeting angiogenesis. In contrast, until recently, the presence and role of nerves in the tumor microenvironment have received little attention. It is established that cancer cells can grow around existing nerves and eventually invade them, in a process called perineural invasion (4). This is generally associated with a poor prognosis and can cause severe pain as demonstrated in pancreatic cancer (5). However, during perineural invasion, nerves are passive, in that they essentially provide a route for cancer cell dissemination. The paradigm change established very recently is that the converse phenomenon, that is, the infiltration of tumors by growing nerves (tumor neoneurogenesis or axonogenesis), has been evidenced and linked to cancer progression.

In a landmark paper, Magnon and colleagues (6) have demonstrated that autonomic nerve sprouting in prostate tumors is essential to prostate cancer progression. Sympathetic and parasympathetic nerves were found to be necessary throughout all phases of prostate cancer development in the mouse. On one hand, the early phases of tumor development were found preventable by sympathectomy or genetic deletion of β-adrenergic receptors, and on the other hand, tumors were also infiltrated by parasympathetic cholinergic fibers that promoted cancer dissemination. Catecholamines and acetylcholine, secreted by sympathetic and parasympathetic nerves, were responsible for the stimulation of prostate tumor growth and metastasis, respectively. Although actions of the sympathetic and parasympathetic nervous systems are classically in opposition, this study suggests that in cancer, they are in fact complementary, where sympathetic nerves stimulate early phases and parasympathetic nerves activate the late metastatic process. Interestingly, stromal cells were found to express β-adrenergic receptors and muscarinic receptors, and to

Published OnlineFirst March 20, 2015; DOI: 10.1158/0008-5472.CAN-14-3180

be targeted by the corresponding ligands, catecholamines, and acetylcholine, secreted by nerves. This reinforces the concept that stromal cells interact with tumor cells and impact their biology. In human prostate tumors, the density of nerves was directly correlated to the Gleason prostate cancer score and tumor aggressiveness, thus providing a clinical relevance to the findings in mice. Taken together, this study was the first to clearly demonstrate that the nervous system is involved in cancer progression and that autonomic nerves are an essential component of the tumor microenvironment, participating in cancer growth and metastasis.

Furthermore, a recent study by Zhao and colleagues (7) has shown that denervation suppresses gastric tumorigenesis. Using a mouse model of gastric cancer, surgical and pharmacologic denervation of the stomach, by vagotomy or local injection of neurotoxic agents, strongly reduced tumor incidence and progression. In addition, denervation was able to enhance the therapeutic effect of systemic chemotherapy and may therefore be a feasible strategy for the control of gastric cancer. In terms of mechanisms, the denervation-induced suppression of gastric tumorigenesis was associated with the inhibition of Wnt signaling and subsequent suppression of stem cell expansion mediated through cholinergic signaling. Cholinergic nerves have been shown to regulate murine gastrointestinal epithelial proliferation (8), and therefore the trophic function of cholinergic nerves for normal gastric epithelial cells appears to extend to the corresponding cancer cells, thus contributing to gastric cancer progression.

Interestingly, it has already been shown that receptors for autonomic neurotransmitters can stimulate cancer cell growth through the activation of corresponding signaling pathways. For instance, the activation of β-adrenergic receptors is essential to malignant growth in ovarian cancer (9) and accelerates pancreatic (10) and pulmonary (11) cancer cell growth and invasion. In addition to their effect on cancer cells, autonomic neurotransmitters are also known to stimulate endothelial cells, immune cells, and fibroblasts (12) and therefore their impact on the tumor microenvironment is likely to be broader and extend beyond a direct stimulation of cancer cells. Lymphocytes express catecholamine receptors and macrophages respond to acetylcholine and in many cases, these interactions are immunosuppressive and anti-inflammatory (13). However, until now the clinical relevance of autonomic neurotransmitters in cancer was unclear as their concentration in the blood is not sufficient to induce a potent effect on cancer cell growth. The discovery of the impact of nerves in prostate and gastric tumors sheds a new light on the role of autonomic neurotransmitters on tumor cell growth, as nerve fibers can release these neurotransmitters directly into the vicinity of cancer cells to stimulate their survival, proliferation, and ability to spread.

Neurogenic Activity of Tumor Cells: The Role of Neurotrophic Growth Factors

Although the two pioneer studies in prostate and gastric cancer revealed the impact of nerves in cancer progression (6, 7), the key drivers of neuron outgrowth in tumors were not identified. However, two recently published studies in prostate cancer have shown that the attraction of nerve fibers is mediated through the production of neurotrophic growth factors by cancer cells. Pundavela and colleagues (14) have shown that proNGF, the precursor of nerve growth factor (NGF), is expressed in prostate cancer cells and a key driver of nerve infiltration. ProNGF was found to be overexpressed in prostate cancer cells compared with normal and benign hyperplastic prostate epithelial cells. ProNGF was correlated to tumor aggressiveness and low-risk tumors (Gleason score $= 6$) contained significantly less proNGF than intermediate and high-risk tumors (Gleason score ≥ 7). Interestingly, although at this stage, there are no supporting mechanistic animal studies, prostate cancer cells *in vitro* were found able to stimulate neuron outgrowth through the secretion of proNGF (14). Whether or not proNGF acts directly on neurons, or is first processed into NGF, is still to be determined, but this study showed that proNGF/NGFs are involved in the neurogenic ability of prostate cancer cells. ProNGF/NGFs have been shown to be involved in perineural invasion (5), and the study by Pundavela and colleagues (14) indicates that these growth factors also participate in tumor neoneurogenesis. Another investigation by Dobrenis and colleagues (15) has shown that the hematopoietic growth factor G-CSF constrains the growth of prostate cancer cells by supporting the survival of sympathetic nerve fibers. Growth factors generally have pleiotropic functions and although G-CSF was not originally described as a neurotrophic molecule, this study shows that it contributes to neoneurogenesis in prostate cancer. Together the studies by Pundavela and colleagues (14) and Dobrenis and colleagues (15) demonstrate that prostate tumors have the ability to attract nerve fibers through the production and release of neurotrophic growth factors. In addition, cancer cells have been shown to secrete axon guidance molecules such as netrins (16, 17), and it could be hypothesized that these molecules also contribute to facilitate nerve infiltration. Overall, neurotrophic growth factor receptors and neurotransmitter receptors (NTR), such as β-adrenergic receptors, are expressed in both neurons and cancer cells, and their corresponding ligands act as messengers between the nervous system and cancer. This opens new perspectives to revisit the role of neurotrophic growth factors and axon guidance molecules in cancer and their potential value as new oncology treatment target. An overview of nerve–cancer cell cross-talk is presented in Fig. 1.

Potential Antineurogenic Therapies in Cancer

From a therapeutic perspective, the studies by Magnon and colleagues (6) and Zhao and colleagues (7) have shown that targeting nerve fibers in prostate and gastric cancer can inhibit tumor growth and metastasis and could therefore be of clinical interest. However, the drugs used in these investigations, such as 6-hydroxydopamine (6-OHDA) and botulinum toxin, as well as other neurotoxic drugs, are unlikely to be of clinical use as they cross the blood brain barrier and are highly toxic to the central nervous system. There are also issues about the specificity as neurotoxic drugs can eventually impact on non-neuronal cells and tissues. Therefore, inhibiting nerve infiltration without inducing neuronal and non-neuronal toxicity is of great importance to future translation of this discovery to the clinic, and the recent identification of neurotrophic factors as drivers of cancer innervation (14, 15) offers a rationale for new therapeutic strategies.

To date, most strategies for targeting neurotrophic factors have been developed for NGF. As NGF plays an important role in generation of pain (18), blocking antibodies, small pharmacologic inhibitors, and peptides have been designed to antagonize this growth factor and its receptors TrkA and $p75^{NTR}$ (19). In

Published OnlineFirst March 20, 2015; DOI: 10.1158/0008-5472.CAN-14-3180

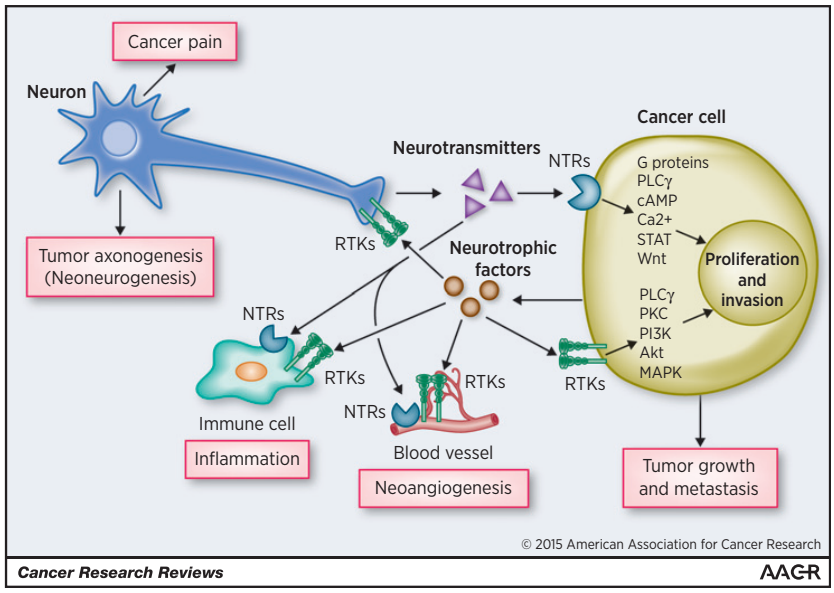


Figure 1.
Nerve–cancer cell cross-talk. Nerves infiltrate the tumor microenvironment and stimulate cancer cell growth and metastasis through the secretion of neurotransmitters (such as catecholamines, acetylcholine, and neuropeptides), initiating signaling pathways for growth and invasion in cancer cells after binding to NTRs. Conversely, nerve infiltration in the tumor is mediated through the liberation of neurotrophic growth factors (such as NGF) by cancer cells, resulting in neuron outgrowth (axonogenesis or neo-neurogenesis), as well as autocrine stimulation of cancer cells via the stimulation of corresponding receptor tyrosine kinases (RTK). This reciprocal interaction fuels tumor development and also impacts the microenvironment, as the liberated neurotransmitters and growth factors can also act on endothelial and immune cells, then contributing to tumor inflammation and neo-angiogenesis. Cancer-induced pain can also be a consequence of tumor innervation. PLCγ, phospholipase C γ; cAMP, cyclic adenosine monophosphate; PKC, protein kinase C.

particular, a humanized monoclonal antibody (tanezumab) is already in clinical trials for its analgesic activity in chronic rheumatoid and back pain. Interestingly, in murine models, anti-NGF antibodies have been shown to decrease pain caused by bone metastases of prostate cancer and to attenuate bone destruction (20, 21). Therefore targeting proNGF/NGF, and more generally neurotrophic factors, could also have an additional positive impact by reducing cancer-associated pain, but this perspective warrants further preclinical and clinical investigations. To date, antineurogenic therapies have been tested for the treatment of pain and neurologic diseases, but they could now be redirected to oncology treatments to inhibit nerve infiltration in cancer. In addition, antineurogenic therapies could have a direct effect on cancer cells, as many cancer cells respond to neurotrophic growth factors, such as NGF, by increasing proliferation and migration via the activation of the corresponding tyrosine kinase receptors. This has been particularly well described for neurotrophins in breast cancer (22). For instance, NGF and proNGF are expressed in breast cancer and stimulate tumor cell growth and invasion (23, 24), including the stem cell compartment (25). In prostate cancer, changes in the expression of NGF and its Trk receptor contribute to tumor cell growth and dissemination through a variety of kinase-based signaling pathways, and the inhibition of Trk receptors decreases the growth of prostatic cancer cells (26). Although neurotrophic factors are less studied in gastric cancer, a recent investigation has reported that the expression of brain-derived neurotrophic growth factor and its receptor TrkB in gastric cancer cells strongly contributes to gastric cancer cell proliferation and dissemination (27). Therefore, targeting neurotrophic growth factors in cancer would have an impact on tumor progression via the inhibition of nerve infiltration, but also through a direct targeting of cancer cells.

Future Directions

Following this major advance, many questions are being generated by the discovery of nerve involvement in prostate and gastric cancers. How generalizable is nerve involvement in cancer progression? The microenvironment of colorectal cancer for instance is rich in autonomic nerve fibers and the presence of nerves has recently been associated with shortened patient survival in colon cancer (28). However, the potential impact of nerves on colorectal cancer progression has not been reported and therefore it is still to be determined whether a similar nerve-dependent tumor growth takes place. In breast cancer, it has been shown that the sympathetic nervous system can induce a metastatic switch (29), but a possible relationship with nerve fiber infiltration in breast tumors has yet to be established. Another potentially important question relates to the impact of β-blockers on survival of patients with cancer. It has been suggested, mainly in breast (30) and in prostate cancer (31), that blockers of the β-adrenergic receptor, traditionally used for the treatment of cardiovascular disorders and anxiety, might increase cancer patient survival. However, the mechanism is not resolved and it

Published OnlineFirst March 20, 2015; DOI: 10.1158/0008-5472.CAN-14-3180

may be hypothesized that beta-blockers actually inhibit the stimulatory effect of catecholamines liberated by nerves in the tumor microenvironment. In addition, the effect of beta-blockers may also be related to the inhibition of stress, as dopamine, a stress inhibitory catecholamine has been shown to decrease ovarian cancer growth though an antiangiogenic effect (32, 33). Finally, to date, only sympathetic and parasympathetic nerves have been implicated in tumor progression and the role of sensory nerves has not yet been reported. Sensory nerves can eventually innervate primary tumors and metastases, thus contributing to tumor-associated pain as demonstrated in pancreatic (5) and prostate cancers (21).Therefore, a possible involvement of sensory fibers in tumor progression, although not demonstrated at this stage, cannot be excluded.

Conclusion

The recent developments described in this review demonstrate that the interaction between nerves and cancer cells goes far beyond the concept of perineural invasion and pain. Nerve infiltration in the tumor microenvironment plays an essential role in both stimulating cancer cell growth and metastasis. These advances bridge a critical gap in knowledge regarding the interplay between the nervous system and cancer, thus opening the way to future investigation of the neurobiology of cancer. The nerve–cancer cell cross-talk opens new therapeutic perspectives in oncology and future development of antineurogenic strategies should be eagerly anticipated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by University of Newcastle Australia and Hunter Cancer Research Alliance.

Received October 27, 2014; revised November 30, 2014; accepted December 16, 2014; published OnlineFirst March 20, 2015.

References

1. De Sousa Pereira A. A basis for sympathectomy for cancer of the cervix uteri. Arch Surg 1946;52:260–85.
2. Barnbrook DH. Pheochromocytoma; an alternative surgical approach. Can Med Assoc J 1953;68:245–7.
3. Batkin S, Piette LH, Wildman E. Effect of muscle denervation on growth of transplanted tumor in mice. Proc Natl Acad Sci U S A 1970;67: 1521–7.
4. Marchesi F, Piemonti L, Mantovani A, Allavena P. Molecular mechanisms of perineural invasion, a forgotten pathway of dissemination and metastasis. Cytokine Growth Factor Rev 2010;21:77–82.
5. Bapat AA, Hostetter G, Von Hoff DD, Han H. Perineural invasion and associated pain in pancreatic cancer. Nat Rev Cancer 2011;11:695–707.
6. Magnon C, Hall SJ, Lin J, Xue X, Gerber L, Freedland SJ, et al. Autonomic nerve development contributes to prostate cancer progression. Science 2013;341:1236361.
7. Zhao CM, Hayakawa Y, Kodama Y, Muthupalani S, Westphalen CB, Andersen GT, et al. Denervation suppresses gastric tumorigenesis. Sci Transl Med 2014;6:250ra115.
8. Gross ER, Gershon MD, Margolis KG, Gertsberg ZV, Li Z, Cowles RA. Neuronal serotonin regulates growth of the intestinal mucosa in mice. Gastroenterology 2012;143:408–17 e2.
9. Thaker PH, Han LY, Kamat AA, Arevalo JM, Takahashi R, Lu C, et al. Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. Nat Med 2006;12:939–44.
10. Kim-Fuchs C, Le CP, Pimentel MA, Shackleford D, Ferrari D, Angst E, et al. Chronic stress accelerates pancreatic cancer growth and invasion: a critical role for beta-adrenergic signaling in the pancreatic microenvironment. Brain Behav Immun 2014;40:40–7.
11. Melamed R, Rosenne E, Shakhar K, Schwartz Y, Abudarham N, Ben-Eliyahu S. Marginating pulmonary-NK activity and resistance to experimental tumor metastasis: suppression by surgery and the prophylactic use of a beta-adrenergic antagonist and a prostaglandin synthesis inhibitor. Brain Behav Immun 2005;19:114–26.
12. Li ZJ, Cho CH. Neurotransmitters, more than meets the eye–neurotransmitters and their perspectives in cancer development and therapy. Eur J Pharmacol 2011;667:17–22.
13. Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, et al. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. Nature 2003;421:384–8.
14. Pundavela J, Demont Y, Jobling P, Lincz LF, Roselli S, Thorne RF, et al. ProNGF correlates with Gleason score and is a potential driver of nerve infiltration in prostate cancer. Am J Pathol 2014;184:3156–62.
15. Dobrenis K, Gauthier LR, Barroca V, Magnon C. Granulocyte colony-stimulating factor off-target effect on nerve outgrowth promotes prostate cancer development. Int J Cancer 2015; 136:982–8.
16. Mehlen P, Delloye-Bourgeois C, Chedotal A. Novel roles for Slits and netrins: axon guidance cues as anticancer targets? Nat Rev Cancer 2011;11:188–97.
17. Biankin AV, Waddell N, Kassahn KS, Gingras MC, Muthuswamy LB, Johns AL, et al. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. Nature 2012;491:399–405.
18. Pezet S, McMahon SB. Neurotrophins: mediators and modulators of pain. Annu Rev Neurosci 2006;29:507–38.
19. Longo FM, Massa SM. Small-molecule modulation of neurotrophin receptors: a strategy for the treatment of neurological disease. Nat Rev Drug Discov 2013;12:507–25.
20. Jimenez-Andrade JM, Ghilardi JR, Castaneda-Corral G, Kuskowski MA, Mantyh PW. Preventive or late administration of anti-NGF therapy attenuates tumor-induced nerve sprouting, neuroma formation, and cancer pain. Pain 2011;152:2564–74.
21. McCaffrey G, Thompson ML, Majuta L, Fealk MN, Chartier S, Longo G, et al. NGF blockade at early times during bone cancer development attenuates bone destruction and increases limb use. Cancer Research 2014;74:7014–23.
22. Hondermarck H. Neurotrophins and their receptors in breast cancer. Cytokine Growth Factor Rev 2012;23:357–65.
23. Adriaenssens E, Vanhecke E, Saule P, Mougel A, Page A, Romon R, et al. Nerve growth factor is a potential therapeutic target in breast cancer. Cancer Res 2008;68:346–51.
24. Demont Y, Corbet C, Page A, Ataman-Onal Y, Choquet-Kastylevsky G, Fliniaux I, et al. Pro-nerve growth factor induces autocrine stimulation of breast cancer cell invasion through tropomyosin-related kinase A (TrkA) and sortilin protein. J. Biol Chem 2012;287:1923–31.
25. Tomellini E, Touil Y, Lagadec C, Julien S, Ostyn P, Ziental-Gelus N, et al. NGF and proNGF simultaneously promote symmetric self-renewal, quiescence and EMT to enlarge the breast cancer stem cell compartment. Stem Cells 2015;33:342–53.
26. Miknyoczki SJ, Wan W, Chang H, Dobrzanski P, Ruggeri BA, Dionne CA, et al. The neurotrophin-trk receptor axes are critical for the growth and progression of human prostatic carcinoma and pancreatic ductal adenocarcinoma xenografts in nude mice. Clin Cancer Res 2002;8: 1924–31.
27. Okugawa Y, Tanaka K, Inoue Y, Kawamura M, Kawamoto A, Hiro J, et al. Brain-derived neurotrophic factor/tropomyosin-related kinase B pathway in gastric cancer. Br J Cancer 2013;108:121–30.
28. Liebl F, Demir IE, Rosenberg R, Boldis A, Yildiz E, Kujundzic K, et al. The severity of neural invasion is associated with shortened survival in colon cancer. Clinical Cancer Research 2013;19:50–61.
29. Sloan EK, Priceman SJ, Cox BF, Yu S, Pimentel MA, Tangkananguku V, et al. The sympathetic nervous system induces a

Published OnlineFirst March 20, 2015; DOI: 10.1158/0008-5472.CAN-14-3180

metastatic switch in primary breast cancer. Cancer Res 2010;70: 7042–52.
30. Barron TI, Connolly RM, Sharp L, Bennett K, Visvanathan K. Beta blockers and breast cancer mortality: a population- based study. J. Clin Oncol 2011;29:2635–44.
31. Grytli HH, Fagerland MW, Fossa SD, Tasken KA. Association between use of beta-blockers and prostate cancer-specific survival: a cohort study of 3561 prostate cancer patients with high-risk or metastatic disease. European Urology 2014;65:635–41.
32. Moreno-Smith M, Lu C, Shahzad MM, Pena GN, Allen JK, Stone RL, et al. Dopamine blocks stress-mediated ovarian carcinoma growth. Clin Cancer Res 2011;17:3649–59.
33. Moreno-Smith M, Lee SJ, Lu C, Nagaraja AS, He G, Rupaimoole R, et al. Biologic effects of dopamine on tumor vasculature in ovarian carcinoma. Neoplasia 2013;15:502–10.
34. Li X, Ma G, Ma Q, Li W, Liu J, Han L, et al. Neurotransmitter substance P mediates pancreatic cancer perineural invasion via NK-1R in cancer cells. Mol Cancer Res 2013;11:294–302.

BIBLIOGRAPHY

Adriaenssens, E., Vanhecke, E., Saule, P., Mougel, A., Page, A., Romon, R., Nurcombe, V., Le Bourhis, X., Hondermarck, H., 2008. Nerve growth factor is a potential therapeutic target in breast cancer. Cancer Res 68, 346-351.

Albo, D., Akay, C.L., Marshall, C.L., Wilks, J.A., Verstovsek, G., Liu, H., Agarwal, N., Berger, D.H., Ayala, G.E., 2011. Neurogenesis in colorectal cancer is a marker of aggressive tumor behavior and poor outcomes. Cancer 117, 4834-4845.

Angeletti, R.H., Bradshaw, R.A., 1971. Nerve growth factor from mouse submaxillary gland: amino acid sequence. Proceedings of the National Academy of Sciences of the United States of America 68, 2417-2420.

Ayala, G.E., Dai, H., Powell, M., Li, R., Ding, Y., Wheeler, T.M., Shine, D., Kadmon, D., Thompson, T., Miles, B.J., Ittmann, M.M., Rowley, D., 2008. Cancer-related axonogenesis and neurogenesis in prostate cancer. Clin Cancer Res 14, 7593-7603.

Bandyopadhyay, D., Mandal, M., Adam, L., Mendelsohn, J., Kumar, R., 1998. Physical interaction between epidermal growth factor receptor and DNA-dependent protein kinase in mammalian cells. The Journal of biological chemistry 273, 1568-1573.

Bapat, A.A., Hostetter, G., Von Hoff, D.D., Han, H., 2011. Perineural invasion and associated pain in pancreatic cancer. Nat Rev Cancer 11, 695-707.

Barde, Y.A., Edgar, D., Thoenen, H., 1982. Purification of a new neurotrophic factor from mammalian brain. The EMBO journal 1, 549-553.

Barnbrook, D.H., 1953. Pheochromocytoma; an alternative surgical approach. Canadian Medical Association journal 68, 245-247.

Barron, T.I., Connolly, R.M., Sharp, L., Bennett, K., Visvanathan, K., 2011. Beta blockers and breast cancer mortality: a population- based study. Journal of Clinical Oncology 29, 2635-2644.

Batkin, S., Piette, L.H., Wildman, E., 1970. Effect of muscle denervation on growth of transplanted tumor in mice. Proceedings of the National Academy of Sciences of the United States of America 67, 1521-1527.

Berger, E.A., Shooter, E.M., 1977. Evidence for pro-beta-nerve growth factor, a biosynthetic precursor to beta-nerve growth factor. Proceedings of the National Academy of Sciences of the United States of America 74, 3647-3651.

Bhattacherjee, A., Liao, Z., Smith, P.G., 2014. Trophic factor and hormonal regulation of neurite outgrowth in sensory neuron-like 50B11 cells. Neuroscience letters 558, 120-12

Biankin, A.V., Waddell, N., Kassahn, K.S., Gingras, M.C., Muthuswamy, L.B., Johns, A.L., Miller, D.K., Wilson, P.J., Patch, A.M., Wu, J., Chang, D.K., Cowley, M.J., Gardiner, B.B., Song, S., Harliwong, I., Idrisoglu, S., Nourse, C., Nourbakhsh, E., Manning, S., Wani, S., Gongora, M., Pajic, M., Scarlett, C.J., Gill, A.J., Pinho, A.V., Rooman, I., Anderson, M., Holmes, O., Leonard, C., Taylor, D., Wood, S., Xu, Q., Nones, K., Fink, J.L., Christ, A., Bruxner, T., Cloonan, N., Kolle, G., Newell, F., Pinese, M., Mead, R.S., Humphris, J.L., Kaplan, W., Jones, M.D., Colvin, E.K., Nagrial, A.M., Humphrey, E.S., Chou, A., Chin, V.T., Chantrill, L.A., Mawson, A., Samra, J.S., Kench, J.G., Lovell, J.A., Daly, R.J., Merrett, N.D., Toon, C., Epari, K., Nguyen, N.Q., Barbour, A., Zeps, N., Australian Pancreatic Cancer Genome, I., Kakkar, N., Zhao, F., Wu, Y.Q., Wang, M., Muzny, D.M., Fisher, W.E., Brunicardi, F.C., Hodges, S.E., Reid, J.G., Drummond, J., Chang, K., Han, Y., Lewis, L.R., Dinh, H., Buhay, C.J., Beck, T., Timms, L., Sam, M., Begley, K., Brown, A., Pai, D., Panchal, A., Buchner, N., De Borja, R., Denroche, R.E., Yung, C.K., Serra, S., Onetto, N., Mukhopadhyay, D., Tsao, M.S., Shaw, P.A., Petersen, G.M., Gallinger, S., Hruban, R.H., Maitra, A., Iacobuzio-Donahue, C.A., Schulick, R.D., Wolfgang, C.L., Morgan, R.A., Lawlor, R.T., Capelli, P., Corbo, V., Scardoni, M., Tortora, G., Tempero, M.A., Mann, K.M., Jenkins, N.A., Perez-Mancera, P.A., Adams, D.J., Largaespada, D.A., Wessels, L.F., Rust, A.G., Stein, L.D., Tuveson, D.A., Copeland, N.G., Musgrove, E.A., Scarpa, A., Eshleman, J.R., Hudson, T.J., Sutherland, R.L., Wheeler, D.A., Pearson, J.V., McPherson, J.D., Gibbs, R.A., Grimmond, S.M., 2012. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. Nature 491, 399-405.

Biarc, J., Chalkley, R.J., Burlingame, A.L., Bradshaw, R.A., 2011. Receptor tyrosine kinase signaling--a proteomic perspective. Advances in enzyme regulation 51, 293-305.

Biarc, J., Chalkley, R.J., Burlingame, A.L., Bradshaw, R.A., 2012. The induction of serine/threonine protein phosphorylations by a PDGFR/TrkA chimera in stably transfected PC12 cells. Molecular & cellular proteomics : MCP 11, 15-30.

Biarc, J., Chalkley, R.J., Burlingame, A.L., Bradshaw, R.A., 2013. Dissecting the roles of tyrosines 490 and 785 of TrkA protein in the induction of downstream protein phosphorylation using chimeric receptors. The Journal of biological chemistry 288, 16606-16618.

Bloom, A.P., Jimenez-Andrade, J.M., Taylor, R.N., Castaneda-Corral, G., Kaczmarska, M.J., Freeman, K.T., Coughlin, K.A., Ghilardi, J.R., Kuskowski, M.A., Mantyh, P.W., 2011. Breast cancer-induced bone remodeling, skeletal pain, and sprouting of sensory nerve fibers. The journal of pain : official journal of the American Pain Society 12, 698-711.

Blume-Jensen, P., Hunter, T., 2001. Oncogenic kinase signalling. Nature 411, 355-365.

Boutilier, J., Ceni, C., Pagdala, P.C., Forgie, A., Neet, K.E., Barker, P.A., 2008. Proneurotrophins require endocytosis and intracellular proteolysis to induce TrkA activation. The Journal of biological chemistry 283, 12709-12716.

Bradshaw, R.A., Chalkley, R.J., Biarc, J., Burlingame, A.L., 2013. Receptor tyrosine kinase signaling mechanisms: Devolving TrkA responses with phosphoproteomics. Advances in biological regulation 53, 87-96.

Campbell, J.P., Karolak, M.R., Ma, Y., Perrien, D.S., Masood-Campbell, S.K., Penner, N.L., Munoz, S.A., Zijlstra, A., Yang, X., Sterling, J.A., Elefteriou, F., 2012. Stimulation of host bone marrow stromal cells by sympathetic nerves promotes breast cancer bone metastasis in mice. PLoS biology 10, e1001363.

Cantarella, G., Lempereur, L., Presta, M., Ribatti, D., Lombardo, G., Lazarovici, P., Zappala, G., Pafumi, C., Bernardini, R., 2002. Nerve growth factor-endothelial cell interaction leads to angiogenesis in vitro and in vivo. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 16, 1307-1309.

Cattaneo, A., 2010. Tanezumab, a recombinant humanized mAb against nerve growth factor for the treatment of acute and chronic pain. Curr Opin Mol Ther 12, 94-106.

Chao, M.V., 2003. Neurotrophins and their receptors: a convergence point for many signalling pathways. Nature reviews. Neuroscience 4, 299-309.

Chao, M.V., Bothwell, M.A., Ross, A.H., Koprowski, H., Lanahan, A.A., Buck, C.R., Sehgal, A., 1986. Gene transfer and molecular cloning of the human NGF receptor. Science 232, 518-521.

Choudhary, C., Mann, M., 2010. Decoding signalling networks by mass spectrometry-based proteomics. Nature reviews. Molecular cell biology 11, 427-439.

Clewes, O., Fahey, M.S., Tyler, S.J., Watson, J.J., Seok, H., Catania, C., Cho, K., Dawbarn, D., Allen, S.J., 2008. Human ProNGF: biological effects and binding profiles at TrkA, P75NTR and sortilin. Journal of neurochemistry 107, 1124-1135.

Com, E., Lagadec, C., Page, A., El Yazidi-Belkoura, I., Slomianny, C., Spencer, A., Hammache, D., Rudkin, B.B., Hondermarck, H., 2007. Nerve growth factor receptor TrkA signaling in breast cancer cells involves Ku70 to prevent apoptosis. Molecular & cellular proteomics : MCP 6, 1842-1854.

Davidson, B., Reich, R., Lazarovici, P., Ann Florenes, V., Nielsen, S., Nesland, J.M., 2004. Altered expression and activation of the nerve growth factor receptors TrkA and p75 provide the first evidence of tumor progression to effusion in breast carcinoma. Breast cancer research and treatment 83, 119-128.

De Sousa Pereira, A., 1946. A basis for sympathectomy for cancer of the cervix uteri. Archives of surgery 52, 260-285.

Delsite, R., Djakiew, D., 1999. Characterization of nerve growth factor precursor protein expression by human prostate stromal cells: a role in selective neurotrophin stimulation of prostate epithelial cell growth. The Prostate 41, 39-48.

Demont, Y., Corbet, C., Page, A., Ataman-Onal, Y., Choquet-Kastylevsky, G., Fliniaux, I., Le Bourhis, X., Toillon, R.A., Bradshaw, R.A., Hondermarck, H., 2012. Pro-nerve growth factor induces autocrine stimulation of breast cancer cell invasion through tropomyosin-related kinase A (TrkA) and sortilin protein. Journal of Biological Chemistry 287, 1923-1931.

Descamps, S., Lebourhis, X., Delehedde, M., Boilly, B., Hondermarck, H., 1998. Nerve growth factor is mitogenic for cancerous but not normal human breast epithelial cells. The Journal of biological chemistry 273, 16659-16662.

Descamps, S., Toillon, R.A., Adriaenssens, E., Pawlowski, V., Cool, S.M., Nurcombe, V., Le Bourhis, X., Boilly, B., Peyrat, J.P., Hondermarck, H., 2001. Nerve growth factor stimulates proliferation and survival of human breast cancer cells through two distinct signaling pathways. The Journal of biological chemistry 276, 17864-17870.

Djakiew, D., Delsite, R., Pflug, B., Wrathall, J., Lynch, J.H., Onoda, M., 1991. Regulation of growth by a nerve growth factor-like protein which modulates paracrine interactions between a neoplastic epithelial cell line and stromal cells of the human prostate. Cancer Res 51, 3304-3310.

Djakiew, D., Pflug, B.R., Delsite, R., Onoda, M., Lynch, J.H., Arand, G., Thompson, E.W., 1993. Chemotaxis and chemokinesis of human prostate tumor cell lines in response to human prostate stromal cell secretory proteins containing a nerve growth factor-like protein. Cancer Res 53, 1416-1420.

Dobrenis, K., Gauthier, L.R., Barroca, V., Magnon, C., 2014. Granulocyte colony-stimulating factor off-target effect on nerve outgrowth promotes prostate cancer development. International Journal of Cancer.

Dolle, L., El Yazidi-Belkoura, I., Adriaenssens, E., Nurcombe, V., Hondermarck, H., 2003. Nerve growth factor overexpression and autocrine loop in breast cancer cells. Oncogene 22, 5592-5601.

Drake, J.M., Lee, J.K., Witte, O.N., 2014. Clinical targeting of mutated and wild-type protein tyrosine kinases in cancer. Molecular and cellular biology 34, 1722-1732.

El Yazidi-Belkoura, I., Adriaenssens, E., Dolle, L., Descamps, S., Hondermarck, H., 2003. Tumor necrosis factor receptor-associated death domain protein is involved in the neurotrophin receptor-mediated antiapoptotic activity of nerve growth factor in breast cancer cells. The Journal of biological chemistry 278, 16952-16956.

Entschladen, F., Drell, T.L.t., Lang, K., Joseph, J., Zaenker, K.S., 2004. Tumour-cell migration, invasion, and metastasis: navigation by neurotransmitters. Lancet Oncol 5, 254-258.

Entschladen, F., Palm, D., Lang, K., Drell, T.L.t., Zaenker, K.S., 2006. Neoneurogenesis: tumors may initiate their own innervation by the release of neurotrophic factors in analogy to lymphangiogenesis and neoangiogenesis. Medical hypotheses 67, 33-35.

Epstein, J.I., Allsbrook, W.C., Jr., Amin, M.B., Egevad, L.L., 2005. The 2005 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma. The American journal of surgical pathology 29, 1228-1242.

Fahnestock, M., Michalski, B., Xu, B., Coughlin, M.D., 2001. The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer's disease. Mol Cell Neurosci 18, 210-220.

Fahnestock, M., Yu, G., Michalski, B., Mathew, S., Colquhoun, A., Ross, G.M., Coughlin, M.D., 2004. The nerve growth factor precursor proNGF exhibits neurotrophic activity but is less active than mature nerve growth factor. Journal of neurochemistry 89, 581-592.

Frazier, W.A., Angeletti, R.H., Bradshaw, R.A., 1972. Nerve growth factor and insulin. Science 176, 482-488.

Garcia-Recio, S., Fuster, G., Fernandez-Nogueira, P., Pastor-Arroyo, E.M., Park, S.Y., Mayordomo, C., Ametller, E., Mancino, M., Gonzalez-Farre, X., Russnes, H.G., Engel, P., Costamagna, D., Fernandez, P.L., Gascon, P., Almendro, V., 2013. Substance P autocrine signaling contributes to persistent HER2 activation that drives malignant progression and drug resistance in breast cancer. Cancer Research 73, 6424-6434.

Gimbrone, M.A., Jr., Leapman, S.B., Cotran, R.S., Folkman, J., 1972. Tumor dormancy in vivo by prevention of neovascularization. The Journal of experimental medicine 136, 261-276.

Glinka, Y., Gassen, M., Youdim, M.B., 1997. Mechanism of 6-hydroxydopamine neurotoxicity. J Neural Transm Suppl 50, 55-66.

Gnad, F., Gunawardena, J., Mann, M., 2011. PHOSIDA 2011: the posttranslational modification database. Nucleic acids research 39, D253-260.

Gross, E.R., Gershon, M.D., Margolis, K.G., Gertsberg, Z.V., Li, Z., Cowles, R.A., 2012. Neuronal serotonin regulates growth of the intestinal mucosa in mice. Gastroenterology 143, 408-417 e402.

Grytli, H.H., Fagerland, M.W., Fossa, S.D., Tasken, K.A., 2014. Association between use of beta-blockers and prostate cancer-specific survival: a cohort study of 3561 prostate cancer patients with high-risk or metastatic disease. European Urology 65, 635-641.

Halvorson, K.G., Kubota, K., Sevcik, M.A., Lindsay, T.H., Sotillo, J.E., Ghilardi, J.R., Rosol, T.J., Boustany, L., Shelton, D.L., Mantyh, P.W., 2005. A blocking antibody to nerve growth factor attenuates skeletal pain induced by prostate tumor cells growing in bone. Cancer Res 65, 9426-9435.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646-674.

Harburg, G.C., Hinck, L., 2011. Navigating breast cancer: axon guidance molecules as breast cancer tumor suppressors and oncogenes. Journal of mammary gland biology and neoplasia 16, 257-270.

Hempstead, B.L., 2014. Deciphering proneurotrophin actions. Handbook of experimental pharmacology 220, 17-32.

Hernandez-Bedolla, M.A., Carretero-Ortega, J., Valadez-Sanchez, M., Vazquez-Prado, J., Reyes-Cruz, G., 2015. Chemotactic and proangiogenic role of calcium sensing receptor is linked to secretion of multiple cytokines and growth factors in breast cancer MDA-MB-231 cells. Biochimica et biophysica acta 1853, 166-182.

Hondermarck, H., 2012. Neurotrophins and their receptors in breast cancer. Cytokine and Growth Factor Reviews 23, 357-365.

Howard, L., Wyatt, S., Nagappan, G., Davies, A.M., 2013. ProNGF promotes neurite growth from a subset of NGF-dependent neurons by a p75NTR-dependent mechanism. Development 140, 2108-2117.

Huang, D., Su, S., Cui, X., Shen, X., Zeng, Y., Wu, W., Chen, J., Chen, F., He, C., Liu, J., Huang, W., Liu, Q., Su, F., Song, E., Ouyang, N., 2014. Nerve fibers in breast cancer tissues indicate aggressive tumor progression. Medicine 93, e172.

Ip, N.Y., Ibanez, C.F., Nye, S.H., McClain, J., Jones, P.F., Gies, D.R., Belluscio, L., Le Beau, M.M., Espinosa, R., 3rd, Squinto, S.P., et al., 1992. Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. Proceedings of the National Academy of Sciences of the United States of America 89, 3060-3064.

Jimenez-Andrade, J.M., Ghilardi, J.R., Castaneda-Corral, G., Kuskowski, M.A., Mantyh, P.W., 2011. Preventive or late administration of anti-NGF therapy attenuates tumor-induced nerve sprouting, neuroma formation, and cancer pain. Pain 152, 2564-2574.

Jobling, P., Pundavela, J., Oliveira, S.M., Roselli, S., Walker, M.M., Hondermarck, H., 2015. Nerve-Cancer Cell Cross-talk: A Novel Promoter of Tumor Progression. Cancer Res 75, 1777-1781.

Kalous, A., Nangle, M.R., Anastasia, A., Hempstead, B.L., Keast, J.R., 2012. Neurotrophic actions initiated by proNGF in adult sensory neurons may require peri-somatic glia to drive local cleavage to NGF. Journal of neurochemistry 122, 523-536.

Kang, J., Perry, J.K., Pandey, V., Fielder, G.C., Mei, B., Qian, P.X., Wu, Z.S., Zhu, T., Liu, D.X., Lobie, P.E., 2009. Artemin is oncogenic for human mammary carcinoma cells. Oncogene 28, 2034-2045.

Kaplan, D.R., Hempstead, B.L., Martin-Zanca, D., Chao, M.V., Parada, L.F., 1991. The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science 252, 554-558.

Kaplan, D.R., Miller, F.D., 2000. Neurotrophin signal transduction in the nervous system. Current opinion in neurobiology 10, 381-391.

Karak, S.G., Quatrano, N., Buckley, J., Ricci, A., Jr., 2010. Prevalence and significance of perineural invasion in invasive breast carcinoma. Connecticut medicine 74, 17-21.

Kim-Fuchs, C., Le, C.P., Pimentel, M.A., Shackleford, D., Ferrari, D., Angst, E., Hollande, F., Sloan, E.K., 2014. Chronic stress accelerates pancreatic cancer growth and invasion: a critical role for beta-adrenergic signaling in the pancreatic microenvironment. Brain, behavior, and immunity 40, 40-47.

Klein, R., Jing, S.Q., Nanduri, V., O'Rourke, E., Barbacid, M., 1991. The trk proto-oncogene encodes a receptor for nerve growth factor. Cell 65, 189-197.

Kouchalakos, R.N., Bradshaw, R.A., 1986. Nerve growth factor receptor from rabbit sympathetic ganglia membranes. Relationship between subforms. The Journal of biological chemistry 261, 16054-16059.

Krebs, E.G., Fischer, E.H., 1964. Phosphorylase and Related Enzymes of Glycogen Metabolism. Vitamins and hormones 22, 399-410.

Kruttgen, A., Schneider, I., Weis, J., 2006. The dark side of the NGF family: neurotrophins in neoplasias. Brain pathology 16, 304-310.

Lagadec, C., Meignan, S., Adriaenssens, E., Foveau, B., Vanhecke, E., Romon, R., Toillon, R.A., Oxombre, B., Hondermarck, H., Le Bourhis, X., 2009. TrkA overexpression enhances growth and metastasis of breast cancer cells. Oncogene 28, 1960-1970.

Lagadec, C., Romon, R., Tastet, C., Meignan, S., Com, E., Page, A., Bidaux, G., Hondermarck, H., Le Bourhis, X., 2010. Ku86 is important for TrkA overexpression-induced breast cancer cell invasion. Proteomics. Clinical applications 4, 580-590.

Lee, R., Kermani, P., Teng, K.K., Hempstead, B.L., 2001. Regulation of cell survival by secreted proneurotrophins. Science 294, 1945-1948.

Leon, A., Buriani, A., Dal Toso, R., Fabris, M., Romanello, S., Aloe, L., Levi-Montalcini, R., 1994. Mast cells synthesize, store, and release nerve growth factor. Proceedings of the National Academy of Sciences of the United States of America 91, 3739-3743.

Levi-Montalcini, R., 1987. The nerve growth factor 35 years later. Science 237, 1154-1162.

Ley, S., Weigert, A., Weichand, B., Henke, N., Mille-Baker, B., Janssen, R.A., Brune, B., 2013. The role of TRKA signaling in IL-10 production by apoptotic tumor cell-activated macrophages. Oncogene 32, 631-640.

Li, X., Ma, G., Ma, Q., Li, W., Liu, J., Han, L., Duan, W., Xu, Q., Liu, H., Wang, Z., Sun, Q., Wang, F., Wu, E., 2013. Neurotransmitter substance P mediates pancreatic cancer perineural invasion via NK-1R in cancer cells. Mol Cancer Res 11, 294-302.

Li, Z.J., Cho, C.H., 2011. Neurotransmitters, more than meets the eye--neurotransmitters and their perspectives in cancer development and therapy. European Journal of Pharmacology 667, 17-22.

Liebl, F., Demir, I.E., Rosenberg, R., Boldis, A., Yildiz, E., Kujundzic, K., Kehl, T., Dischl, D., Schuster, T., Maak, M., Becker, K., Langer, R., Laschinger, M., Friess, H., Ceyhan, G.O., 2013. The severity of neural invasion is associated with shortened survival in colon cancer. Clinical Cancer Research 19, 50-61.

Lobos, E., Gebhardt, C., Kluge, A., Spanel-Borowski, K., 2005. Expression of nerve growth factor (NGF) isoforms in the rat uterus during pregnancy: accumulation of precursor proNGF. Endocrinology 146, 1922-1929.

Longo, F.M., Massa, S.M., 2013. Small-molecule modulation of neurotrophin receptors: a strategy for the treatment of neurological disease. Nat Rev Drug Discov 12, 507-525.

Luthy, I.A., Bruzzone, A., Pinero, C.P., Castillo, L.F., Chiesa, I.J., Vazquez, S.M., Sarappa, M.G., 2009. Adrenoceptors: non conventional target for breast cancer? Current medicinal chemistry 16, 1850-1862.

Magnon, C., Hall, S.J., Lin, J., Xue, X., Gerber, L., Freedland, S.J., Frenette, P.S., 2013. Autonomic nerve development contributes to prostate cancer progression. Science 341, 1236361.

Maisonpierre, P.C., Le Beau, M.M., Espinosa, R., 3rd, Ip, N.Y., Belluscio, L., de la Monte, S.M., Squinto, S., Furth, M.E., Yancopoulos, G.D., 1991. Human and rat brain-derived neurotrophic factor and neurotrophin-3: gene structures, distributions, and chromosomal localizations. Genomics 10, 558-568.

Marchesi, F., Piemonti, L., Mantovani, A., Allavena, P., 2010. Molecular mechanisms of perineural invasion, a forgotten pathway of dissemination and metastasis. Cytokine and Growth Factor Reviews 21, 77-82.

Masoudi, R., Ioannou, M.S., Coughlin, M.D., Pagadala, P., Neet, K.E., Clewes, O., Allen, S.J., Dawbarn, D., Fahnestock, M., 2009. Biological activity of nerve growth factor precursor is dependent upon relative levels of its receptors. The Journal of biological chemistry 284, 18424-18433.

McCaffrey, G., Thompson, M.L., Majuta, L., Fealk, M.N., Chartier, S., Longo, G., Mantyh, P., 2014. NGF blockade at early times during bone cancer development attenuates bone destruction and increases limb use. Cancer Research.

Mehlen, P., Delloye-Bourgeois, C., Chedotal, A., 2011. Novel roles for Slits and netrins: axon guidance cues as anticancer targets? Nat Rev Cancer 11, 188-197.

Melamed, R., Rosenne, E., Shakhar, K., Schwartz, Y., Abudarham, N., Ben-Eliyahu, S., 2005. Marginating pulmonary-NK activity and resistance to experimental tumor metastasis: suppression by surgery and the prophylactic use of a beta-adrenergic antagonist and a prostaglandin synthesis inhibitor. Brain, behavior, and immunity 19, 114-126.

Melhem-Bertrandt, A., Chavez-Macgregor, M., Lei, X., Brown, E.N., Lee, R.T., Meric-Bernstam, F., Sood, A.K., Conzen, S.D., Hortobagyi, G.N., Gonzalez-Angulo, A.M., 2011. Beta-blocker use is associated with improved relapse-free survival in patients with triple-negative breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 29, 2645-2652.

Miknyoczki, S.J., Wan, W., Chang, H., Dobrzanski, P., Ruggeri, B.A., Dionne, C.A., Buchkovich, K., 2002. The neurotrophin-trk receptor axes are critical for the growth and progression of human prostatic carcinoma and pancreatic ductal adenocarcinoma xenografts in nude mice. Clin Cancer Res 8, 1924-1931.

Moreno-Smith, M., Lee, S.J., Lu, C., Nagaraja, A.S., He, G., Rupaimoole, R., Han, H.D., Jennings, N.B., Roh, J.W., Nishimura, M., Kang, Y., Allen, J.K., Armaiz, G.N., Matsuo, K., Shahzad, M.M., Bottsford-Miller, J., Langley, R.R., Cole, S.W., Lutgendorf, S.K., Siddik, Z.H., Sood, A.K., 2013. Biologic effects of dopamine on tumor vasculature in ovarian carcinoma. Neoplasia 15, 502-510.

Moreno-Smith, M., Lu, C., Shahzad, M.M., Pena, G.N., Allen, J.K., Stone, R.L., Mangala, L.S., Han, H.D., Kim, H.S., Farley, D., Berestein, G.L., Cole, S.W., Lutgendorf, S.K., Sood, A.K., 2011. Dopamine blocks stress-mediated ovarian carcinoma growth. Clin Cancer Res 17, 3649-3659.

Nilsson, G., Forsberg-Nilsson, K., Xiang, Z., Hallbook, F., Nilsson, K., Metcalfe, D.D., 1997. Human mast cells express functional TrkA and are a source of nerve growth factor. European journal of immunology 27, 2295-2301.

Nykjaer, A., Lee, R., Teng, K.K., Jansen, P., Madsen, P., Nielsen, M.S., Jacobsen, C., Kliemannel, M., Schwarz, E., Willnow, T.E., Hempstead, B.L., Petersen, C.M., 2004. Sortilin is essential for proNGF-induced neuronal cell death. Nature 427, 843-848.

Okugawa, Y., Tanaka, K., Inoue, Y., Kawamura, M., Kawamoto, A., Hiro, J., Saigusa, S., Toiyama, Y., Ohi, M., Uchida, K., Mohri, Y., Kusunoki, M., 2013. Brain-derived neurotrophic factor/tropomyosin-related kinase B pathway in gastric cancer. British Journal of Cancer 108, 121-130.

Olsen, J.V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., Mann, M., 2006. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 127, 635-648.

Ondicova, K., Mravec, B., 2010. Role of nervous system in cancer aetiopathogenesis. Lancet Oncol 11, 596-601.

Pawson, T., 2002. Regulation and targets of receptor tyrosine kinases. European journal of cancer 38 Suppl 5, S3-10.

Penault-Llorca, F., Bertucci, F., Adelaide, J., Parc, P., Coulier, F., Jacquemier, J., Birnbaum, D., deLapeyriere, O., 1995. Expression of FGF and FGF receptor genes in human breast cancer. International journal of cancer. Journal international du cancer 61, 170-176.

Pezet, S., McMahon, S.B., 2006. Neurotrophins: mediators and modulators of pain. Annu Rev Neurosci 29, 507-538.

Powe, D.G., Voss, M.J., Habashy, H.O., Zanker, K.S., Green, A.R., Ellis, I.O., Entschladen, F., 2011. Alpha- and beta-adrenergic receptor (AR) protein expression is associated with poor clinical outcome in breast cancer: an immunohistochemical study. Breast cancer research and treatment 130, 457-463.

Pundavela, J., Demont, Y., Jobling, P., Lincz, L.F., Roselli, S., Thorne, R.F., Bond, D., Bradshaw, R.A., Walker, M.M., Hondermarck, H., 2014. ProNGF Correlates with Gleason Score and Is a Potential Driver of Nerve Infiltration in Prostate Cancer. The American journal of pathology 184, 3156-3162.

Radeke, M.J., Misko, T.P., Hsu, C., Herzenberg, L.A., Shooter, E.M., 1987. Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325, 593-597.

Raffioni, S., Bradshaw, R.A., Buxser, S.E., 1993. The receptors for nerve growth factor and other neurotrophins. Annual review of biochemistry 62, 823-850.

Reichardt, L.F., 2006. Neurotrophin-regulated signalling pathways. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 361, 1545-1564.

Romon, R., Adriaenssens, E., Lagadec, C., Germain, E., Hondermarck, H., Le Bourhis, X., 2010. Nerve growth factor promotes breast cancer angiogenesis by activating multiple pathways. Molecular cancer 9, 157.

Sarhadi, N.S., Shaw Dunn, J., Lee, F.D., Soutar, D.S., 1996. An anatomical study of the nerve supply of the breast, including the nipple and areola. British journal of plastic surgery 49, 156-164.

Schlessinger, J., 2000. Cell signaling by receptor tyrosine kinases. Cell 103, 211-225.

Schmidt, B.L., Hamamoto, D.T., Simone, D.A., Wilcox, G.L., 2010. Mechanism of cancer pain. Molecular interventions 10, 164-178.

Scott, J., Selby, M., Urdea, M., Quiroga, M., Bell, G.I., Rutter, W.J., 1983. Isolation and nucleotide sequence of a cDNA encoding the precursor of mouse nerve growth factor. Nature 302, 538-540.

Seidah, N.G., Benjannet, S., Pareek, S., Chretien, M., Murphy, R.A., 1996. Cellular processing of the neurotrophin precursors of NT3 and BDNF by the mammalian proprotein convertases. FEBS letters 379, 247-250.

Shooter, E.M., 2001. Early days of the nerve growth factor proteins. Annu Rev Neurosci 24, 601-629.

Skaper, S.D., 2012. The neurotrophin family of neurotrophic factors: an overview. Methods in Molecular Biology 846, 1-12.

Sloan, E.K., Priceman, S.J., Cox, B.F., Yu, S., Pimentel, M.A., Tangkanangnukul, V., Arevalo, J.M., Morizono, K., Karanikolas, B.D., Wu, L., Sood, A.K., Cole, S.W., 2010. The sympathetic nervous system induces a metastatic switch in primary breast cancer. Cancer Res 70, 7042-7052.

Sortino, M.A., Condorelli, F., Vancheri, C., Chiarenza, A., Bernardini, R., Consoli, U., Canonico, P.L., 2000. Mitogenic effect of nerve growth factor (NGF) in LNCaP prostate adenocarcinoma cells: role of the high- and low-affinity NGF receptors. Mol Endocrinol 14, 124-136.

Suter, D.M., Miller, K.E., 2011. The emerging role of forces in axonal elongation. Progress in neurobiology 94, 91-101.

Swartz, M.A., Iida, N., Roberts, E.W., Sangaletti, S., Wong, M.H., Yull, F.E., Coussens, L.M., DeClerck, Y.A., 2012. Tumor microenvironment complexity: emerging roles in cancer therapy. Cancer Res 72, 2473-2480.

Teng, K.K., Felice, S., Kim, T., Hempstead, B.L., 2010. Understanding proneurotrophin actions: Recent advances and challenges. Dev Neurobiol 70, 350-359.

Thaker, P.H., Han, L.Y., Kamat, A.A., Arevalo, J.M., Takahashi, R., Lu, C., Jennings, N.B., Armaiz-Pena, G., Bankson, J.A., Ravoori, M., Merritt, W.M., Lin, Y.G., Mangala, L.S., Kim, T.J., Coleman, R.L., Landen, C.N., Li, Y., Felix, E., Sanguino, A.M., Newman, R.A., Lloyd, M., Gershenson, D.M., Kundra, V., Lopez-Berestein, G., Lutgendorf, S.K., Cole, S.W., Sood, A.K., 2006. Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. Nature medicine 12, 939-944.

Tomellini, E., Touil, Y., Lagadec, C., Julien, S., Ostyn, P., Ziental-Gelus, N., Meignan, S., Lengrand, J., Adriaenssens, E., Polakowska, R., Le Bourhis, X., 2014. NGF and proNGF simultaneously promote symmetric self-renewal, quiescence and EMT to enlarge the breast cancer stem cell compartment. Stem cells.

Tomita, T., 2012. Localization of nerve fibers in colonic polyps, adenomas, and adenocarcinomas by immunocytochemical staining for PGP 9.5. Digestive diseases and sciences 57, 364-370.

Truzzi, F., Marconi, A., Lotti, R., Dallaglio, K., French, L.E., Hempstead, B.L., Pincelli, C., 2008. Neurotrophins and their receptors stimulate melanoma cell proliferation and migration. Journal of Investigative Dermatology 128, 2031-2040.

Ullrich, A., Gray, A., Berman, C., Dull, T.J., 1983. Human beta-nerve growth factor gene sequence highly homologous to that of mouse. Nature 303, 821-825.

Vanhecke, E., Adriaenssens, E., Verbeke, S., Meignan, S., Germain, E., Berteaux, N., Nurcombe, V., Le Bourhis, X., Hondermarck, H., 2011. Brain-derived neurotrophic factor and neurotrophin-4/5 are expressed in breast cancer and can be targeted to inhibit tumor cell survival. Clinical Cancer Research 17, 1741-1752.

Verbeke, S., Meignan, S., Lagadec, C., Germain, E., Hondermarck, H., Adriaenssens, E., Le Bourhis, X., 2010. Overexpression of p75(NTR) increases survival of breast cancer cells through p21(waf1). Cellular signalling 22, 1864-1873.

Villers, A., McNeal, J.E., Redwine, E.A., Freiha, F.S., Stamey, T.A., 1989. The role of perineural space invasion in the local spread of prostatic adenocarcinoma. The Journal of urology 142, 763-768.

Wang, H., Yu, M., Ochani, M., Amella, C.A., Tanovic, M., Susarla, S., Li, J.H., Wang, H., Yang, H., Ulloa, L., Al-Abed, Y., Czura, C.J., Tracey, K.J., 2003. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. Nature 421, 384-388.

Weeraratna, A.T., Dalrymple, S.L., Lamb, J.C., Denmeade, S.R., Miknyoczki, S., Dionne, C.A., Isaacs, J.T., 2001. Pan-trk inhibition decreases metastasis and enhances host survival in experimental models as a result of its selective induction of apoptosis of prostate cancer cells. Clin Cancer Res 7, 2237-2245.

Zeidan, Q., Hart, G.W., 2010. The intersections between O-GlcNAcylation and phosphorylation: implications for multiple signaling pathways. Journal of cell science 123, 13-22.

Zhao, C.M., Hayakawa, Y., Kodama, Y., Muthupalani, S., Westphalen, C.B., Andersen, G.T., Flatberg, A., Johannessen, H., Friedman, R.A., Renz, B.W., Sandvik, A.K., Beisvag, V., Tomita, H., Hara, A., Quante, M., Li, Z., Gershon, M.D., Kaneko, K., Fox, J.G., Wang, T.C., Chen, D., 2014a. Denervation suppresses gastric tumorigenesis. Sci Transl Med 6, 250ra115.

Zhao, Q., Yang, Y., Liang, X., Du, G., Liu, L., Lu, L., Dong, J., Han, H., Zhang, G., 2014b. The clinicopathological significance of neurogenesis in breast cancer. BMC cancer 14, 484.

ADDENDUM

A.1 ADDITIONAL PUBLICATION:

The published paper entitled "**Sortilin is associated with Breast Cancer Aggressiveness and Contributes to Tumor Cell Adhesion and Invasion**" is a supplementary paper to this thesis, which examined the role of the proNGF receptor sortilin in breast cancer. This is published in the journal *Oncotarget.*

www.impactjournals.com/oncotarget/ Oncotarget, Vol. 6, No. 12

Sortilin is associated with breast cancer aggressiveness and contributes to tumor cell adhesion and invasion

Séverine Roselli[1,2], Jay Pundavela[1,2], Yohann Demont[3,5], Sam Faulkner[1,2], Sheridan Keene[1,2], John Attia[2,4], Chen Chen Jiang[1,2], Xu Dong Zhang[1,2], Marjorie M. Walker[2,4], Hubert Hondermarck[1,2]

[1]School of Biomedical Sciences & Pharmacy, Faculty of Health and Medicine, University of Newcastle, Callaghan NSW 2308, Australia

[2]Hunter Medical Research Institute, University of Newcastle, New Lambton NSW 2305, Australia

[3]INSERM U908, IFR-147, Universite Lille 1, Villeneuve d'Ascq 59655, France

[4]School of Public Health & Medicine, Faculty of Health and Medicine, University of Newcastle, Callaghan NSW 2308, Australia

[5]INSERM U1138, Equipe 11, Centre de Recherche des Cordeliers, Paris 75006, France

Correspondence to:
Hubert Hondermarck, **e-mail:** hubert.hondermarck@newcastle.edu.au

Keywords: *breast cancer, sortilin, protein expression, cell adhesion, cell invasion*

Received: *January 21, 2015* ***Accepted:*** *February 16, 2015* ***Published:*** *March 18, 2015*

ABSTRACT

The neuronal membrane protein sortilin has been reported in a few cancer cell lines, but its expression and impact in human tumors is unclear. In this study, sortilin was analyzed by immunohistochemistry in a series of 318 clinically annotated breast cancers and 53 normal breast tissues. Sortilin was detected in epithelial cells, with increased levels in cancers, as compared to normal tissues ($p = 0.0088$). It was found in 79% of invasive ductal carcinomas and 54% of invasive lobular carcinomas ($p < 0.0001$). There was an association between sortilin expression and lymph node involvement ($p = 0.0093$), suggesting a relationship with metastatic potential. In cell culture, sortilin levels were higher in cancer cell lines compared to non-tumorigenic breast epithelial cells and siRNA knockdown of sortilin inhibited cancer cell adhesion, while proliferation and apoptosis were not affected. Breast cancer cell migration and invasion were also inhibited by sortilin knockdown, with a decrease in focal adhesion kinase and SRC phosphorylation. In conclusion, sortilin participates in breast tumor aggressiveness and may constitute a new therapeutic target against tumor cell invasion.

INTRODUCTION

The expression of nervous system related proteins in cancer is an intriguing feature of several carcinomas that probably stems from the shared developmental origin of neurons and epithelial cells, which both derive from the neuroepithelial layer of the embryo. Neurotrophic growth factors [1], neuronal guidance molecules [2] or receptors for neurotransmitters [3] are expressed in tumors and, similarly to their role in the nervous system, may contribute to the plasticity of cancer cells.

Sortilin is a neuronal type-1 membrane protein, encoded by the *SORT1* gene, that belongs to the Vacuolar Protein Sorting 10 protein (VPS10P) family of receptors and is most abundantly expressed in both the central and peripheral nervous systems [4]. Sortilin is composed of a transmembrane segment and a short cytoplasmic tail, including motifs for interaction with cytosolic adaptor molecules. Initially described as the neurotensin receptor-3, sortilin is more generally involved in protein sorting and trafficking via a complex pattern whereby it shuttles between the cell surface and various intracellular compartments, directing target proteins to distinct destinations [5]. It is a common binding partner of tyrosine kinase receptors, G-protein coupled receptors and ion-channels, for which it facilitates ligand-induced signalling [6]. Sortilin has been identified as a co-receptor for neurotensin and pro-nerve growth factor (proNGF), and in the latter case acts in a

complex with the neurotrophin receptor p75^{NTR} to induce neuron apoptosis [6, 7]. Further to its neuronal death-promoting activity, sortilin has also recently been identified as a receptor for apolipoprotein E and is a key factor in the catabolism of amyloid-β peptide in the brain [8]. Overall, sortilin is an essential regulator of neuronal viability and a potential therapeutic target in neurodegenerative diseases, but its role outside the nervous system, and particularly in cancer remains to be determined.

In non-neuronal tissues, sortilin expression has been reported in skeletal and heart muscles, adrenal gland, thyroid, lymphocyte B cells as well as keratinocytes and adipocytes [9–12]. A few cancer cell lines have been shown to express sortilin and are impacted by its disruption. In the HT29 colon cancer cells, sortilin participates in the control of growth promoting activity by brain-derived growth factor, through interacting with its tyrosine kinase receptor TrkB [13]. Additionally, sortilin mediates the release and transfer of exosomes in the A549 lung cancer cell line [14]. In prostate cancer cells, sortilin has been shown to regulate progranulin stimulatory activity of cancer cell growth [15]. In melanoma cell lines, sortilin is a co-receptor for pro-nerve growth factor (proNGF), and acts in cooperation with the neurotrophin receptor p75^{NTR} to promote cancer cell invasion [16]. Similarly, in breast cancer cell lines sortilin has been shown to participate in proNGF induced-cell invasion through cooperation with the tyrosine kinase receptor TrkA [17]. Together, data about the impact of sortilin in cancer are fragmentary, and as the expression of sortilin has never been reported in a cohort of human cancers, its clinicopathological significance in oncology is unclear.

In the present study, sortilin protein levels were analyzed by immunohistochemistry in a cohort of clinically annotated breast cancers and normal breast tissues. The expression of sortilin was found increased in breast cancer, particularly in ductal invasive carcinomas, and there was an association with lymph node invasion. In addition, decreasing sortilin protein level resulted in a diminished adhesion and invasion of breast cancer cells.

RESULTS

Sortilin protein expression in breast cancers

Sortilin was analyzed by immunohistochemistry in a series of 318 clinically annotated breast cancers and 53 adjacent normal tissues. Sortilin expression was found only in epithelial cells of both normal and cancerous samples (Fig. 1). No labeling was observed in the stroma: fibroblasts, endothelial cells, adipocytes and extracellular matrix were all negative. The frequency distribution of sortilin levels (Fig. 2) showed that the majority of normal tissues had low levels of sortilin (staining intensity 0 and 1), while the proportion of cases with intermediate (staining intensity 2) and high (staining intensity 3) levels of sortilin increased in cancers and in particular in invasive ductal carcinomas (IDC) and lymph node positive tumors. There was a clear difference between sortilin positive and sortilin negative cases (Fig. 1) and among sortilin positive cases, the staining intensities were fairly homogeneous (mostly staining intensities 1 and 2). Therefore, the data were expressed in terms of sortilin positive *versus* sortilin negative cancer cases (Table 1). Analysis of relationships between sortilin expression and clinicopathological parameters revealed sortilin expression in 66% of breast cancers compared to 47% of adjacent normal tissues ($p = 0.0088$). A difference in expression between invasive ductal carcinomas (IDC) and invasive lobular carcinomas (ILC) was observed: 79% of IDC were positive for sortilin as compared to 54% of ILC ($p < 0.0001$). No significant association of sortilin expression was observed with tumor size, grade, patient age, ER and PR, and molecular subtypes of breast cancer (luminal A and B, HER2+, triple negative). Sortilin was expressed in 59% of triple negative breast cancers. In addition, there was a trend toward more tumors expressing sortilin among HER2-positive tumors (77%) than among HER2-negative tumors (63%) but the *p*-value was limited ($p = 0.0349$). A significant association was found between sortilin expression and lymph node invasion. Sortilin was expressed in 60% of lymph node negative cancers *versus* 75% of lymph node positive cancers ($p = 0.0093$), suggesting a positive relationship between sortilin expression and the metastatic potential. In Log-Linear modeling, two-way analyses confirmed the association, adjusted for all other variables, of sortilin with histological type (ductal vs. lobular invasive carcinomas, $p = 0.002$) and lymph node invasion (OR = 1.55 for lymph node positivity, $p = 0.096$).

Sortilin expression in breast cancer cell lines

A series of normal, immortalized and cancerous breast epithelial cells was analyzed for sortilin expression by RT-PCR and Western-blotting (Fig. 3). qRT-PCR analysis showed varying levels of sortilin mRNA in normal and cancer cell lines (Fig. 3A). All breast cancer cell lines expressed more mRNA for sortilin than the normal breast epithelial cells (HMEC). In Western-blotting, a band at about 100 kDa, which corresponds to the expected migration of sortilin, was observed in all tested cells (Fig. 3B). In MCF-7, SKBR-3 and BT-474 cells, an additional minor band at 50 kDa was also detected. This additional band may represent a degraded form of sortilin, which requires further characterization. Overall, there was more sortilin in cancer cell lines than in the normal HMEC. In the HMEC-derivatives model of breast carcinogenesis [18], there was an increase of sortilin in the tumorigenic HMLE and HMLER as compared to the normal HMEC and the transformed but non-tumorigenic HME (Fig. 3C) (the entire blot is shown in Supplemental Data).

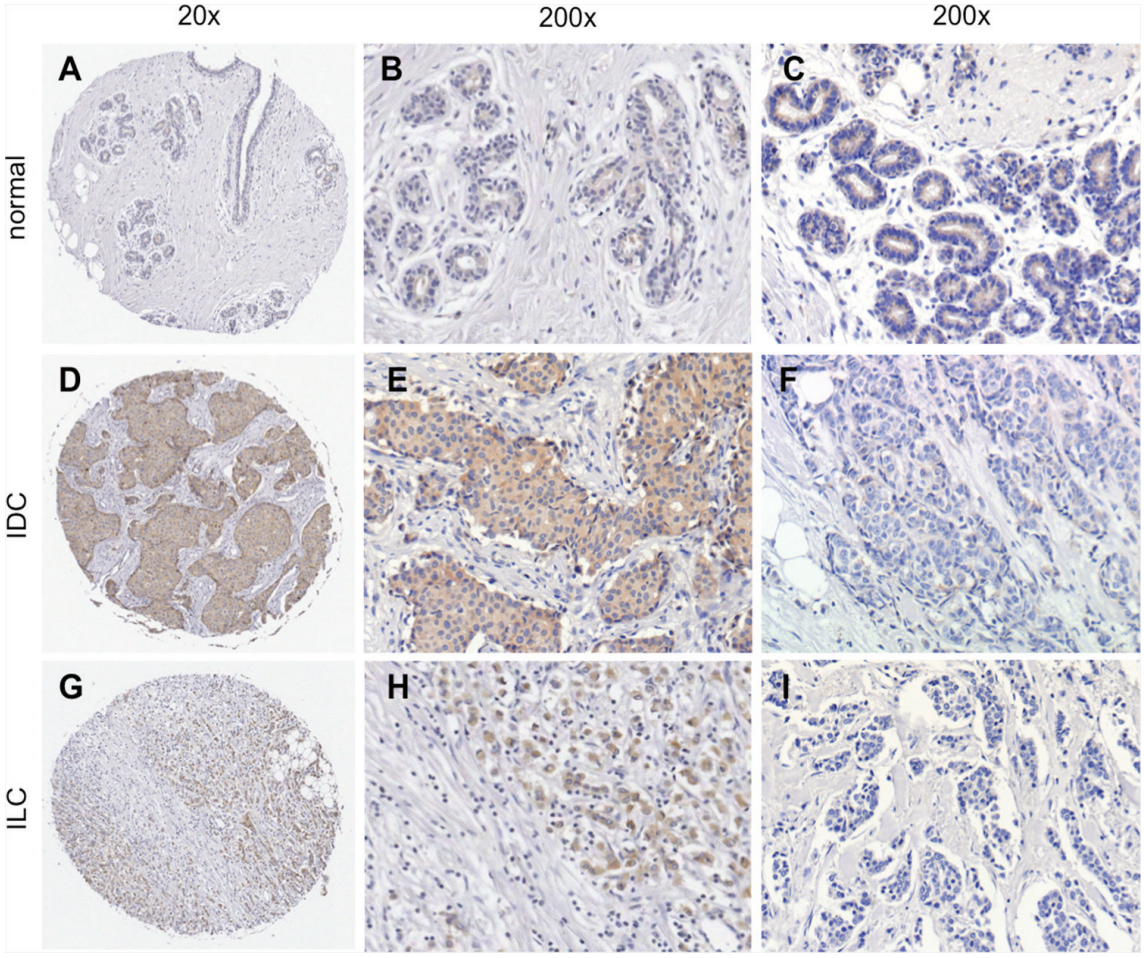


Figure 1: Immunohistological detection of sortilin in breast cancers. The expression of sortilin was assessed by immunohistochemistry in a series of invasive breast cancers and normal adjacent tissues. Representative photos of sortilin immunolabeling are shown. **A.** Entire core and **B, C.** higher magnifications obtained for normal breast adjacent tissue; **D.** Entire core and **E.** higher magnification obtained for an invasive ductal carcinoma (IDC) positive for sortilin; **F.** Sortilin negative IDC. **G.** Entire core and **H.** higher magnification obtained for an invasive lobular carcinoma (ILC) positive for sortilin; **I.** Sortilin negative ILC. Magnification (20x, 200x) is indicated.

Impact of sortilin inhibition on breast cancer cell phenotype

The functional analysis was performed on the highly invasive and triple negative MDA-MB-231 breast cancer cell line, the HER2 overexpressing SKBR-3, and the luminal A type MCF-7 cells. Breast cancer cell lines were transfected with siRNA against sortilin *versus* control siRNA and the impact on cell growth, survival, adhesion, migration and invasion was measured. The efficacy of siRNA was assessed by Western-blotting at 24, 48 and 72 h after transfection (Fig. 4A). In MDA-MB-231 cells, a strong decrease in sortilin protein was observed from 24 h and was maintained after 72 h. In SKBR-3 and MCF-7 cells, the inhibition was complete only at 48 h, but was also maintained at 72 h. Microscopic observation 72 h after transfection (Fig. 4B) suggested that there were fewer cells in siRNA sortilin than in control siRNA, with a lower attachment (higher proportion of round cells). The decrease in cell number was confirmed by cell counting (Fig. 4C). This has prompted us to analyze cell cycle and apoptosis. Flow cytometry after propidium iodide incorporation (Fig. 4D) indicated no change in the proportion of cells in each phase of the cell cycle (G1/G0, S, G2M) between the siRNA sortilin and the siRNA control conditions. This demonstrated that the sortilin siRNA had no impact on cell proliferation. In addition there was also no change

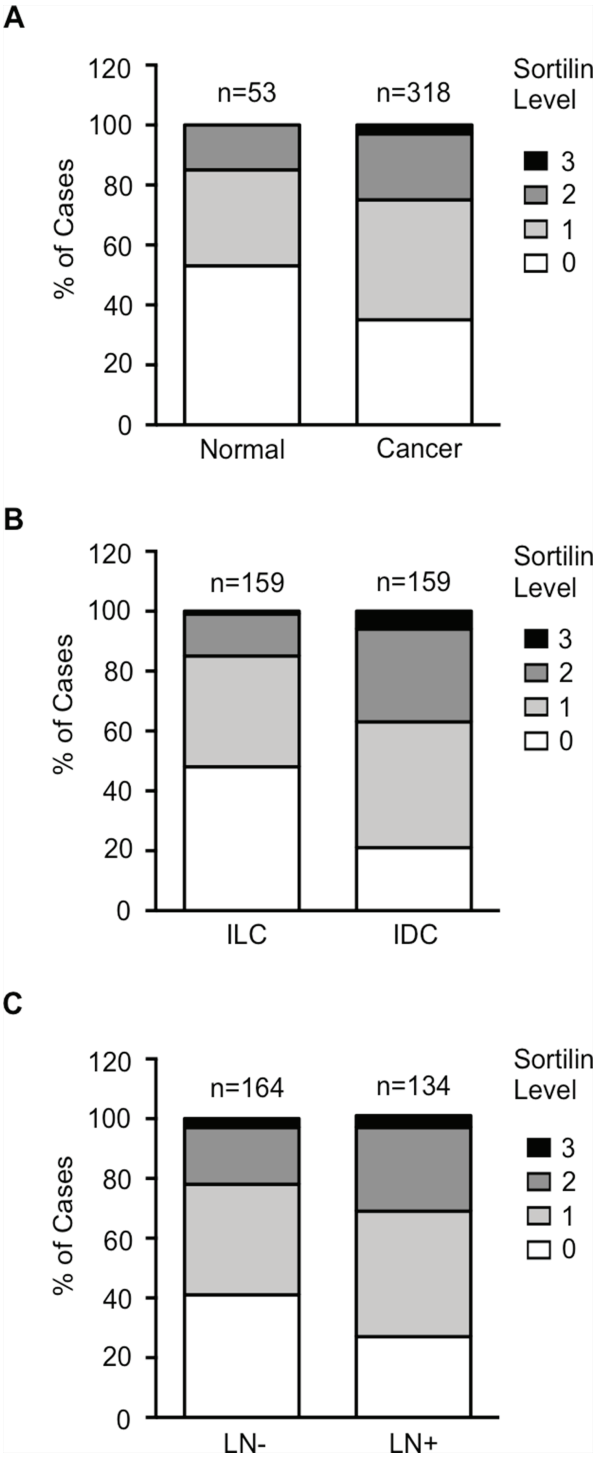


Figure 2: Frequency distribution of sortilin levels. Sortilin levels (0 = no staining, 1 = low intensity staining, 2 = intermediate intensity staining, 3 = high intensity staining) were measured in breast cancers and normal breast tissues. **A.** Distribution in normal tissues versus breast tumors. **B.** Distribution in invasive lobular carcinomas (ILC) versus invasive ductal carcinomas (IDC). **C.** Distribution in lymph node negative (LN-) versus lymph node positive (LN+) cancers. Number of cases (n) is indicated. Statistical significance of the difference between groups are reported in Table 1.

Table 1: Association between sortilin expression and clinicopathological parameters in breast cancer

	Sortilin negative	Sortilin positive	*p*-value
Normal vs cancer			
Normal (*n* = 53)	28 (53%)	25 (47%)	0.0088
Cancer (*n* = 318)	107 (34%)	211 (66%)	
Pathological type			
IDC (*n* = 159)	34 (21%)	125 (79%)	< 0.0001[a]
ILC (*n* = 159)	73 (46%)	86 (54%)	
Age (years)			
< 50 (*n* = 171)	54 (32%)	117 (68%)	0.4075
≥ 50 (*n* = 147)	53 (36%)	94 (64%)	
Tumor size			
T1 (*n* = 25)	11 (44%)	14 (56%)	0.0951
T2 (*n* = 228)	75 (33%)	153 (67%)	
T3 (*n* = 31)	15 (48%)	16 (52%)	
T4 (*n* = 29)	6 (26%)	23 (74%)	
Lymph node status			
LN- (*n* = 164)	65 (40%)	99 (60%)	0.0093[a]
LN+ (*n* = 134)	34 (25%)	100 (75%)	
HER2			
HER2- (*n* = 252)	92 (37%)	160 (63%)	0.0349
HER2+ (*n* = 66)	15 (23%)	51 (77%)	
Estrogen receptor			
ER- (*n* = 182)	67 (37%)	115 (63%)	0.1670
ER+ (*n* = 136)	40 (29%)	96 (71%)	
Progesterone receptor			
PR- (*n* = 208)	75 (36%)	133 (64%)	0.2111
PR+ (*n* = 110)	32 (29%)	78 (71%)	
Breast cancer subtypes			
luminal A (*n* = 129)	44 (34%)	85 (66%)	0.1329
luminal B (*n* = 33)	9 (27%)	24 (73%)	
HER2 (*n* = 33)	7 (21%)	26 (79%)	
TNBC (*n* = 122)	50 (41%)	72 (59%)	

Abbreviations: IDC = Invasive ductal carcinoma; ILC = Invasive lobular carcinomas; HER2 = Human epidermal growth factor receptor 2; ER = estrogen receptor; PR = progesterone receptor; TNBC = Triple negative breast cancer.
Chi-square test was used to test statistical association. Statistically significant *P*-values ($p < 0.05$) are shown in bold.
[a]The association with histological type and lymph node invasion was confirmed by two-way Log-Linear analysis, but not the association with HER2+

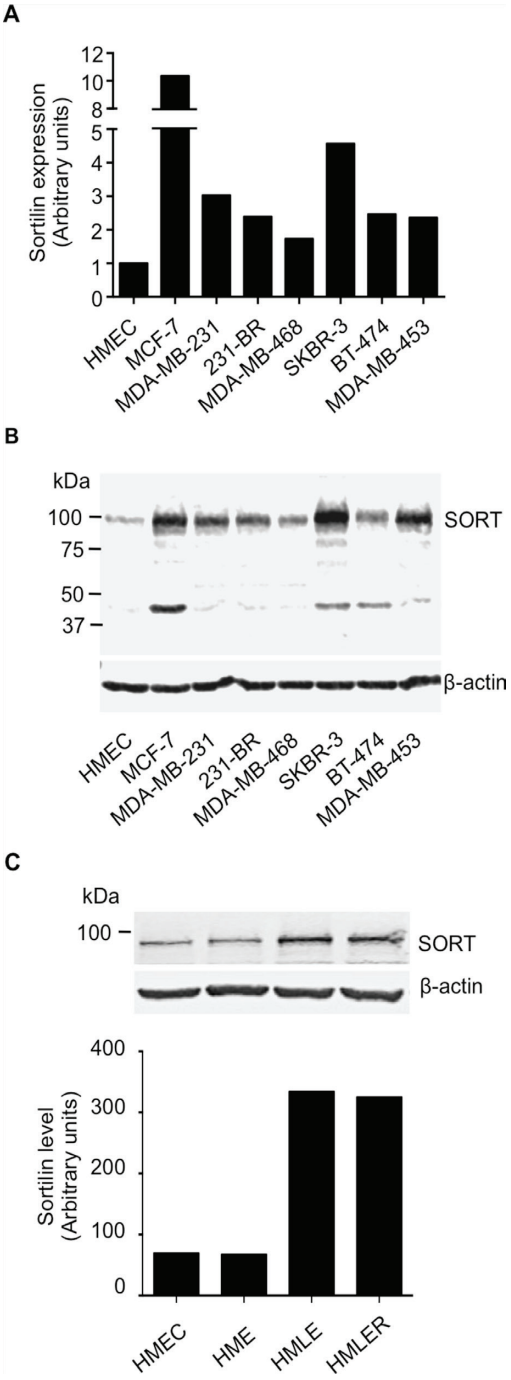


Figure 3: Expression of sortilin in breast cancer cell lines. A. Quantitative RT-PCR analysis of sortilin gene expression in a range of breast cancer cell lines. Human mammary epithelial cells (HMEC) and the breast cancer cell lines MCF-7, MDA-MB-231 and their brain metastatic derivatives 231-BR, SKBR-3, MDA-MB-468, BT-474 and MDA-MB-453 were analyzed. Normalization was performed using β actin and the value obtained for HMEC was considered as 1. **B.** Western-blotting detection of sortilin in the same breast cancer cell lines. A band at about 100 kDa, the expected molecular weight of sortilin, was observed in all cell lines. In MCF-7, SKBR-3 and BT474 cells, an additional band at 50 kDa was also detected. **C.** Sortilin was detected in the HMEC derivatives model of breast tumorigenic progression. The intensity of the sortilin band was higher in the tumorigenic HMLE and HMLER cells compared to the precancerous HME and the normal non-transformed HMEC.

in subG0/G1, suggesting that siRNA against sortilin did not induce cell death. This was confirmed by Hoechst staining (Fig. 4D), as no particular nuclei fragmentation or condensation could be observed in the anti-sortilin siRNA condition. About 5% of apoptosis could be observed for all cell lines with or without anti-sortilin siRNA. Therefore, the decrease in cell number observed after sortilin siRNA transfection was not due to a decrease in cell proliferation or an increase in cell death. This has prompted us to test the impact of the sortilin siRNA on cell adhesion. Interestingly, breast cancer cell adhesion was affected by sortilin siRNA knockdown (Fig. 4E). SiRNA against sortilin resulted in 30% inhibition of MCF-7 cell adhesion, as measured 20 h after cell seeding. In SKBR-3, the inhibition of cell adhesion was ~50% and it reached ~80% in MDA-MB-231 cells. These results indicated that sortilin is involved in breast cancer cell adhesion. We then investigated the impact of sortilin knockdown on breast cancer cell migration and invasion (Fig. 5). In wound healing assay, anti-sortilin siRNA inhibited the migration of MDA-MB-231 cells (Fig. 5A). In contrast the migration of MCF-7 and SKBR-3 cells was not affected (Fig. 5A). We then looked for the invasive property of MDA-MB-231 in Transwell assays. The invasion of MDA-MB-231 cells was significantly inhibited by anti-sortilin siRNA (Fig. 5B). To take under account the potential impact of the inhibition of cell adhesion on the invasion of MDA-MB-231 cells, we counted the number of cells attached in both the upper part and the down-side of the Transwell filters (Fig.5B left panel). We then expressed the percentage (%) of invading cells, as compared to attached cells (Fig. 5B right panel). The results show that the invasion of cancer cells that had attached was inhibited and therefore, siRNA against sortilin had a direct inhibitory effect on MDA-MB-231 cell invasion. We have then explored the level of activation of various cell invasion-related signaling pathways (Fig. 5C) (the entire blots are shown in Supplemental Data). Western-blotting experiments revealed that the level of vimentin was not affected by siRNA against sortilin, indicating that the EMT (epithelial-mesenchymal transition) phenotype of MDA-MB-231 was not altered. Akt and Erk1/2 phosphorylation was also not modified, but in contrast, the activation of SRC and focal adhesion kinase (FAK) was inhibited by anti-sortilin siRNA. Therefore, the sortilin knockdown-induced inhibition of MDA-MB-231 breast cancer cell invasion involves a decrease in SRC/FAK signaling pathways.

DISCUSSION

This study is the first to report sortilin expression in a series of human tumors. The results highlight an increase in sortilin protein level in breast cancer cells, particularly in invasive ductal carcinomas, as well as an association between sortilin and lymph node invasion. Furthermore, the *in vitro* data point to a participation of sortilin in adhesion and invasion of breast cancer cells.

In terms of gene expression, sortilin mRNA abundance has not been reported to be linked to a particular molecular subtype of breast cancer or clinicopathological parameter. Data mining, using cBioportal [19] of The Cancer Genome Atlas (TCGA) database [20], which contains 1062 samples of invasive breast carcinomas, indicated that sortilin is altered in 7.2% of breast tumors with 5 cases of amplification, 2 homozygous deletions, 4 mutations, 59 mRNA up regulations, and 8 mRNA down regulations (data not shown). The 59/1062 cases of mRNA amplifications represented 5.5% of all breast cancer cases. In addition, using the Gene Expression-Based Outcome for Breast cancer Online (GOBO) [21] with datasets GSE1456, 3494, 7390, representing a total of 737 breast cancers, no relationship was found between sortilin mRNA abundance and clinicopathological parameters (molecular subtypes, lymph node invasion, ER, PR, HER2). Initial studies in yeast comparing mRNA *versus* protein levels have suggested a correlation of ~50% between mRNA and protein levels. In humans, global transcriptomic and proteomic analyses have shown that an estimated 30%–60% of changes in protein levels can be explained by corresponding variations in mRNA [23, 24]. In addition, a recent proteogenomics investigation in colorectal cancer [25] has revealed that mRNA abundance does not reliably predict protein abundance differences between tumors. This emphasizes the importance to analyse the protein level, as gene expression data may not reflect the abundance of the protein effectors in tumors.

In the present study, sortilin protein was found in a higher proportion of IDC than ILC. IDC represent the majority of breast cancers (~80%) and are generally more aggressive than ILC [26]. Sortilin expression was also detected across the molecular subtypes of breast tumors (luminal A, luminal B, HER2+ and triple negative/basal) with no significant difference. Interestingly, triple negative breast cancers, which do not express oestrogen receptor, progesterone receptor and the tyrosine kinase receptor HER2, were found to be positive for sortilin in 59% of cases. At this stage, triple negative breast cancers are characterized by what they don't express and they are the only molecular subtype of breast cancers for which there is no targeted therapies [27, 28]. As a consequence, triple negative tumors have a particularly poor prognosis with a higher propensity to metastasize. Our data suggest that sortilin could potentially be targeted in breast cancer, particularly in the aggressive and difficult to treat triple negative tumors.

The increased level of sortilin protein in breast cancers, alongside the association with lymph node invasion, has prompted us to look at the impact of sortilin inhibition in breast cancer cells. Our data indicated that decreasing the level of sortilin diminished breast cancer cell adhesion, while having no effect on cell proliferation

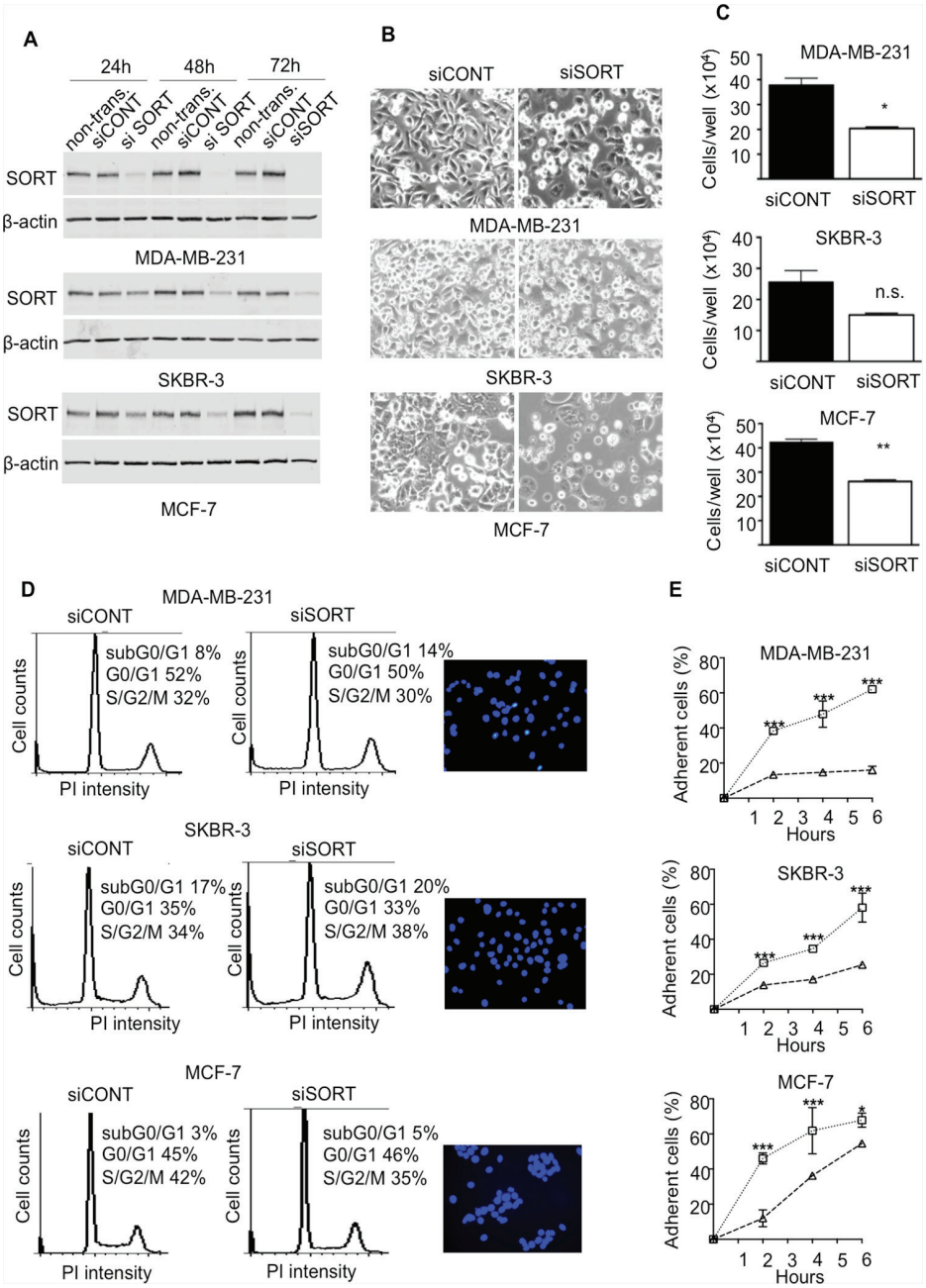


Figure 4: Impact of sortilin knockdown on proliferation, survival and adhesion of breast cancer cells. A. SiRNA against Sortilin (siSORT) and universal negative control siRNA (siCONT) were transfected in MDA-MB-231, MCF-7 and SKBR-3 breast cancer cells, and the impact on the level of sortilin was measured by Western-blotting 24, 48 and 72 h after transfection. Non-transfected cells (non transf.) were also analyzed. **B.** Microscopic observation of breast cancer cells 72 h after transfection with siSORT and siCONT. **C.** Counting of breast cancer cells 72 h after transfection with siSORT versus siCONT. The histograms represent the mean number of cells per well. **D.** Flow cytometry analysis of breast cancer cells 72 h after transfection with siSORT or siCONT. The percentage of cells in SG2M, G0/G1 and subG0/G1 is indicated. For each cell line, a picture of Hoechst staining observed in siSORT is shown. **E.** Impact of siRNA against sortilin on breast cancer cell adhesion. Breast cancer cells were transfected with siRNA and were seeded in culture dishes. 48 h latter, number of attached cells was counted at the indicated times after seeding. Results are expressed, as percentage of adherent cells. For panel C and E, error bars represent SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s. not significant, for the difference between siCONT and siSORT.

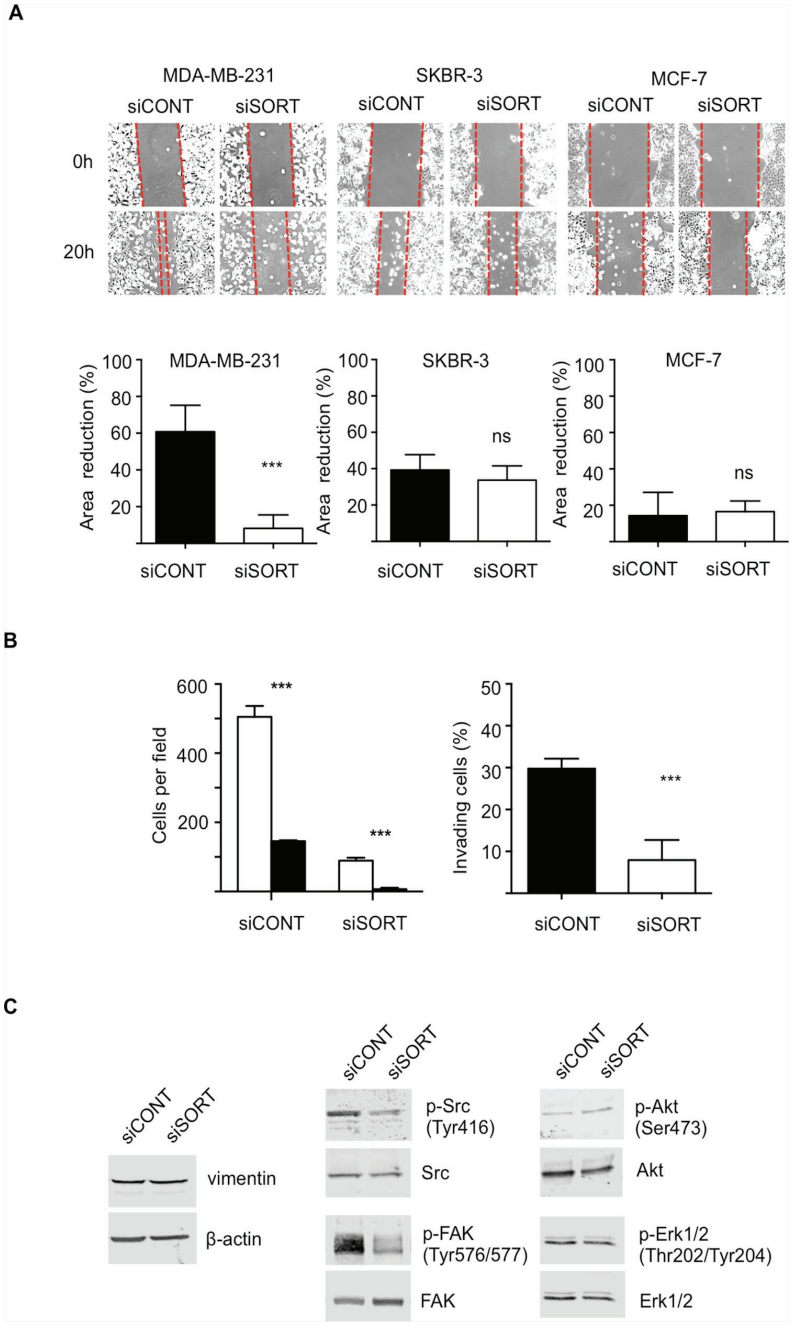


Figure 5: Impact of sortilin knockdown on migration and invasion of breast cancer cells. A. Scratch assay. Breast cancer cells (MDA-MB-231, SKBR-3 and MCF-7) were transfected with siRNA against sortilin (siSORT) and universal negative control siRNA (siCONT). Scratching of the cell layer was performed 48 h after transfection and reduction in gap area was measured over 6 h. SiSORT inhibited migration only in MDA-MB-231 cells. **B.** Transwell invasion assay of MDA-MB-231 cells. Transwell assays were set up 48 h after siRNA transfection and cells were allowed to invade for 48 h. To take under account a potential impact of cell adhesion on the assay, cells were counted on both sides of the Transwell filter. Left panel, white columns represent the number of cells on the upper side of the filter, and the black columns the number of cells on the down side. Right panel, the percentage of invading cells in siSORT *versus* siCONT is represented. **C.** Western-blot detection of vimentin and activation of SRC, FAK, Akt and Erk1/2, 72 h after transfection with siSORT versus siCONT. Antibodies against vimentin, β-actin, SRC, phospho-SRC (Tyr416), FAK, phospho-FAK (Tyr576/577), Akt, phosphor-Akt (Ser473), Erk1/2, phosphor-Erk1/2 were used. For panel A and B, error bars represent SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s. not significant, for the difference between siCONT and siSORT.

and survival. Interestingly, soluble forms of sortilin have already been implicated in cell adhesion. In the colorectal cancer cell line HT29, recent studies have shown that soluble sortilin can regulate FAK-dependent activation of the PI3 kinase pathway [29] and that soluble sortilin impairs cell to cell cohesion [30]. In breast cancer cells, we have not detected any soluble forms of sortilin (data not shown), and the molecular mechanism involved in the inhibition of cell adhesion/invasion remains to be determined. Our study also shows that sortilin is involved in breast cancer cell invasion as knockdown of sortilin in the highly invasive MDA-MB-231 cells was found to inhibit cell invasion. The process of cancer cell invasion requires not only cell migration, but also digestion of the extracellular matrix, and changes in cell adhesion are closely associated to the metastatic process [31]. Circulating cancer cells have to attach to the endothelial barrier to establish new tumoral niches and thus remodelling of cell adhesion and invasion is a hallmark of metastatic cells. The kinases SRC and FAK are generally involved in cancer cell adhesion and invasion, including in breast cancer cells [32]. Activation of SRC and FAK can be initiated by integrins and various tyrosine kinase receptors, and we show here that sortilin knockdown resulted in a decreased activation of these kinases. On the other hand, Akt and Erk1/2 were not affected, showing that sortilin inhibition has a targeted effect on cell invasion-related signaling. Further experiments are necessary to precisely define the cellular proteins directly targeted by sortilin in breast cancer cells. It has previously been shown that sortilin acts as a co-receptor for proNGF and is necessary to induce the activation of the tyrosine kinase receptor TrkA [17]. However, in the present study, proNGF was not added to the culture media and therefore, our data show that the impact of inhibiting sortilin on breast cancer cells goes beyond the regulation of proNGF activity. Although the molecular mechanism of action of sortilin in breast cancer cells, and in particular its direct interacting partners, remain to be elucidated, our data suggest sortilin, as a new potential therapeutic target in breast cancer.

In a broader perspective, it is worth noting that sortilin is also a nociceptor involved in the transmission of pain feeling by sensory neurons [33], and therefore, targeting sortilin in oncology could also inhibit cancer pain. To date, there is no available drug against sortilin, however the synthesis of a first small molecule potentially capable of inhibiting sortilin has recently been described [34] and further developments could lead to clinically relevant inhibitors [35]. As sortilin can induce neuronal apoptosis [6, 7], future sortilin inhibitors are anticipated to promote neuron survival and be of potential value for the treatment of neurodegenerative disease. Our study suggests that the inhibition of sortilin could also potentially be used in oncology to inhibit cancer cell invasion. In any case, the value of sortilin, as a potential target, in breast cancer and in other forms of cancer, warrants further consideration.

MATERIALS AND METHODS

Tumor microarrays

High-density tumor microarrays (TMA) of breast cancer biopsies and normal adjacent tissues were obtained from US Biomax Inc (Rockville, USA). These included 158 invasive ductal carcinomas, 159 invasive lobular carcinomas, and 53 normal adjacent tissues (TMAs Catalogue number BR1921 and BR1921a). Histopathological subtypes were reviewed by a pathologist (MMW). Clinical annotations included age, lymph node status, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) status. This study was approved by the Human Research Ethics Committee of the University of Newcastle Australia.

Immunohistochemistry

After deparaffinization and rehydration, the TMAs were treated for immunohistochemistry as previously described [36]. Primary antibodies were rabbit polyclonal anti-sortilin (Cat ANT-009, Alomone Labs, Jerusalem, Israel) and non-immune rabbit IgG control (Alpha Diagnostic, San Antonio, USA) at 0.8 µg/mL. Sortilin labeling was scored by two independent observers including a pathologist, on a scale ranging from 0 to 3, as follows: 0 (no staining), 1 (low intensity staining), 2 (moderate staining), and 3 (strong staining).

Analysis of associations between sortilin expression and clinicopathological parameters

For the purpose of the analysis, because the labeling was homogeneous among sortilin positive cases, the scores were then grouped into two categories: sortilin negative (score 0) and sortilin positive (scores 1, 2, and 3). Simple unadjusted associations between sortilin and other pathological variables were performed using a chi-squared test. We used log-linear models to adjust the various bivariate associations for other potential confounders. The log linear models provided a Chi-squared test adjusted for all other variables; these included cancer type (lobular vs. ductal), lymph node involvement (yes/no), estrogen receptor positivity (yes/no), progesterone receptor positivity (yes/no), HER2+ (yes/no). The model was specified as a Poisson generalized linear model with a log-link function. Using hierarchical nesting of models we looked at all 3-way then 2-way interactions involving sortilin. Goodness of fit was tested using G2 Chi-squared statistics, as well as AIC and BIC. These models were fitted using SAS (SAS Institute, North Carolina, USA).

Cell culture

Breast cancer cells MCF-7, MDA-MB-231, SKBR-3, MDA-MB-468, MDA-MB-453, BT-474 were obtained from the American Type Culture Collection (ATCC, Manassas, USA). The brain metastatic 231-BR cell line was a generous gift from Dr Barbara Steeg (Bethesda, USA). HMEC (human mammary epithelial cells), as well as their derivatives (HME, HMLE, HMLER), were obtained from Dr Robert Weinberg (Boston, USA). Individual cell line authentication was performed after DNA extraction (Promega kit, catalogue number A1120) and using the GenePrint 10 PCR amplification kit (Promega catalogue number B9510). All cancer and non-tumorigenic cell lines were maintained in RPMI-1640 with 10% (v/v) fetal calf serum (FCS) (JRH Biosciences, St. Louis, USA) and 2 mM L-glutamine in a humidified incubator at 37°C with 5% (v/v) CO_2.

Transfection with siRNA

Cells were transfected with siRNA using lipofectamine RNAiMAX (Life Technologies) according to manufacturer's recommendations. Cells were seeded in 6-well plates and transfected 24 h later with siRNA against sortilin (siSORT CUCUGCUGUUAACACCACC[dT][dT] or a siRNA control sequence commercially available from Sigma (MISSION® siRNA Universal negative control #1). The efficiency of sortilin knockdown was assessed by Western-blotting using anti-sortilin antibody (ANT009, Alomone Labs, Israel). Actin detection (Cat antibody A2066, Sigma-Aldrich, St. Louis, USA) was used, as equiloading control.

Western-blotting

Western-blotting experiments were performed, as previously described [36], with anti-Sortilin (1:500 dilution; Cat ANT-009, Alomone Labs, Jerusalem, Israel) and mouse anti-β-actin (1:5000 dilution; Sigma-Aldrich, St. Louis, USA). Antibodies from Cell Signaling Technology (USA) were also used for SRC (cat 2100), phosphoSRC (Tyr416, cat 2101), FAK (cat 1009), phosphoFAK (Tyr576/577, cat 3281), Erk1/2 (cat 9107), phosphoErk1/2 (Thr202/Tyr204, cat 4370), Akt (cat 9272), phosphoAkt (Ser473, cat 9271), vimentin (cat 5741).

Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated from breast cancer cell lines using the illustra RNAspin Mini RNA Isolation Kit (GE Healthcare Life Sciences, Little Chalfont, UK). Reverse transcription was performed with 1 µg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, USA). Real-time PCR was performed using 2 µl 1/10 cDNA using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, USA). Sortilin Primers were Quantitect Primer Assay QT00073318 (Qiagen, Venlo, Netherlands). The PCR was carried out in a ABI7500 Real-Time PCR System (Applied Biosystems, Thermo Scientific, Waltham, USA) using the following conditions, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds followed by a continuous Melt curve from 65°C to 95°C. Data analysis was performed using the ABI7500 Real-Time Software (Applied Biosystems, Thermo Scientific, Waltham, USA). Relative expression was obtained using the $2^{-\Delta\Delta Ct}$ method.

Flow cytometry

Breast cancer cells (MDA-MB-231, SKBR-3, MCF-7) were collected by trypsinization after 72 h siRNA transfection, pooled with the saved growth media, and pelleted at 500 × g for 5 min. After PBS wash and counting, 10^6 cells were gently resuspended in 400 µL of ice-cold PBS followed by addition of 800 µL ice cold 100% (v/v) ethanol in order to achieve fixation in 66% (v/v) ice cold ethanol at 4°C overnight. On the day of cell cycle analysis, the fixed cell samples in ethanol were equilibrated to room temperature, gently re-suspended and pelleted at 500 × g for 5 min followed by a PBS wash. Labeling was performed by addition of 500 µL of FxCycle Propidium iodide/RNase staining solution (Life Technologies, USA) to each sample and incubation for 15–30 min at room temperature in the dark. Cell cycle analysis was performed with a BD FACSCanto flow cytometer (Becton Dickinson, Sydney, Australia) and the data was analyzed using the WEASEL software (WEHI, Melbourne, Australia). The percentage of cells in the different phases of the cell cycle (G0/G1, S, G2/M) as well as the subG0/G1 (indicative of cell death) was determined.

Hoechst staining

The proportion of cells in apoptosis was determined using Hoechst staining, as previously described [37].

Adhesion assay

Breast cancer cells were transfected with anti-sortilin or control siRNA as indicated above. After 48 h they were detached using trypsin free TrypLE dissociation solution (Invitrogen, Thermo Scientific, Waltham, USA), and seeded at 10^5 cells/mL in 12-well cell Corning culture plates (Corning, USA). After 2, 4, and 6 h, adherent cells were counted under a phase contrast microscope. Adherent cells appeared flat and attached, while non-adherent cells were round and mobile. Counting was performed in 5 random fields per culture dish. The assay was done in triplicate.

Migration assay

Breast cancer cells were seeded in 6-well plate (5×10^5 cells per well) and transfected with anti-sortilin

or control siRNA. After 48 h, the cell monolayer was scratched with a 200 µL pipette tip, rinsed three times with PBS and replaced with media containing 0.1% (v/v) FCS. The gap area that resulted from the scratch was monitored by taking pictures of three random areas using a phase contrast microscope (Zeiss) over 6 h post-scratch. Results are shown, as the percentage reduction of the gap area measured using ImageJ (NIH).

Invasion assay

Cell invasion assays were performed in 12-well Boyden microchambers (Transwell®) with 8 µm pore size membranes. Transwells were first coated with 100 µL of starvation medium with 0.1% (v/v) FCS plus 40 µg of rat-tail collagen I for 1 h at 37°C. Cell loading was done with 100,000 siRNA transfected cells (48 h after transfection) in 400 µL starvation medium with 0.1% (v/v) FCS in the upper chamber whereas 1.6 mL starvation medium with 0.1% (v/v) FCS was placed in the lower chamber. After 20 h of incubation, the Transwell filters were rinsed with PBS and cells at the upper surface of the membrane were gently scraped and removed for counting. Cells having invaded to the down side of the membrane were fixed and stained with 0.1% (w/v) crystal violet before counting (10 fields per membrane) through an inverted microscope.

Statistical analysis

In the cell growth, adhesion, migration and invasion assays, each condition was performed in triplicate and statistical analysis was conducted using GraphPad Prism 6. The results of cell growth, migration and invasion assays were compared using a *t*-test and cell adhesion over time was compared using repeated measure two-way ANOVA.

ACKNOWLEDGMENTS

This work was supported by the University of Newcastle Australia, the Hunter Cancer Research Alliance, and the French National Institute of Health (INSERM). We thank Christopher Oldmeadow from the Clinical Research Design, IT, and Statistical Support (CReDITSS) Unit of the University of Newcastle. Helen Carpenter provided excellent technical assistance.

Authors' contributions

SR carried out the immunohistochemistry and siRNA experiments. MW, who is an experienced pathologist, performed the grading and scoring of TMAs. SR duplicated grading and scoring. SK and JP have done the qRT-PCR and Western-blotting respectively. JP also formatted the Figures of the manuscript. SR and CCJ have performed the flow cytometry analysis. YD carried out some of the functional *in vitro* assay of cell growth, apoptosis and invasion. JA supervised the statistical analyses. HH, XDZ and MMW supervised the study. HH conceived the study and drafted the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Mancino M, Ametller E, Gascon P, Almendro V. The neuronal influence on tumor progression. Biochim Biophys Acta. 2011; 1816:105–118.
2. Mehlen P, Delloye-Bourgeois C, Chedotal A. Novel roles for Slits and netrins: axon guidance cues as anticancer targets? Nat Rev Cancer. 2011; 11:188–197.
3. Li ZJ, Cho CH. Neurotransmitters, more than meets the eye—neurotransmitters and their perspectives in cancer development and therapy. Eur J Pharmacol. 2011; 667:17–22.
4. Willnow TE, Petersen CM, Nykjaer A. VPS10P-domain receptors - regulators of neuronal viability and function. Nature reviews Neuroscience. 2008; 9:899–909.
5. Hermey G. The Vps10p-domain receptor family. Cell Mol Life Sci. 2009; 66:2677–2689.
6. Nykjaer A, Willnow TE. Sortilin: a receptor to regulate neuronal viability and function. Trends in neurosciences. 2012; 35:261–270.
7. Nykjaer A, Lee R, Teng KK, Jansen P, Madsen P, Nielsen MS, Jacobsen C, Kliemannel M, Schwarz E, Willnow TE, Hempstead BL, Petersen CM. Sortilin is essential for proNGF-induced neuronal cell death. Nature. 2004; 427:843–848.
8. Carlo AS, Gustafsen C, Mastrobuoni G, Nielsen MS, Burgert T, Hartl D, Rohe M, Nykjaer A, Herz J, Heeren J, Kempa S, Petersen CM, Willnow TE. The pro-neurotrophin receptor sortilin is a major neuronal apolipoprotein E receptor for catabolism of amyloid-beta peptide in the brain. J Neurosci. 2013; 33:358–370.
9. Nielsen MS, Jacobsen C, Olivecrona G, Gliemann J, Petersen CM. Sortilin/neurotensin receptor-3 binds and mediates degradation of lipoprotein lipase. J Biol Chem. 1999; 274:8832–8836.
10. Kiss M, Dallos A, Kormos B, Santha P, Dobozy A, Husz S, Kemeny L. Sortilin is expressed in cultured human keratinocytes and is regulated by cutaneous neuropeptides. J Invest Dermatol. 2010; 130:2553–2560.

11. Fauchais AL, Lalloue F, Lise MC, Boumediene A, Preud'homme JL, Vidal E, Jauberteau MO. Role of endogenous brain-derived neurotrophic factor and sortilin in B cell survival. J Immunol. 2008; 181:3027–3038.
12. Botta R, Lisi S, Pinchera A, Giorgi F, Marcocci C, Taddei AR, Fausto AM, Bernardini N, Ippolito C, Mattii L, Persani L, de Filippis T, Calebiro D, Madsen P, Petersen CM, Marino M. Sortilin is a putative postendocytic receptor of thyroglobulin. Endocrinology. 2009; 150:509–518.
13. Akil H, Perraud A, Melin C, Jauberteau MO, Mathonnet M. Fine-tuning roles of endogenous brain-derived neurotrophic factor, TrkB and sortilin in colorectal cancer cell survival. PloS one. 2011; 6:e25097.
14. Wilson CM, Naves T, Vincent F, Melloni B, Bonnaud F, Lalloue F, Jauberteau MO. Sortilin mediates the release and transfer of exosomes in concert with two tyrosine kinase receptors. J Cell Sci. 2014; 127:3983–3997.
15. Tanimoto R, Morcavallo A, Terraciano M, Xu SQ, Stefanello M, Buraschi S, Lu KG, Bagley DH, Gomella LG, Scotlandi K, Belfiore A, Iozzo RV, Morrione A. Sortilin regulates progranulin action in castration-resistant prostate cancer cells. Endocrinology. 2015:en20141590; 156:58–70.
16. Truzzi F, Marconi A, Lotti R, Dallaglio K, French LE, Hempstead BL, Pincelli C. Neurotrophins and their receptors stimulate melanoma cell proliferation and migration. J Invest Dermatol. 2008; 128:2031–2040.
17. Demont Y, Corbet C, Page A, Ataman-Onal Y, Choquet-Kastylevsky G, Fliniaux I, Le Bourhis X, Toillon RA, Bradshaw RA, Hondermarck H. Pro-nerve growth factor induces autocrine stimulation of breast cancer cell invasion through tropomyosin-related kinase A (TrkA) and sortilin protein. J Biol Chem. 2012; 287:1923–1931.
18. Elenbaas B, Spirio L, Koerner F, Fleming MD, Zimonjic DB, Donaher JL, Popescu NC, Hahn WC, Weinberg RA. Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. Genes Dev. 2001; 15:50–65.
19. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C, Schultz N. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer discovery. 2012; 2:401–404.
20. Cancer Genome Atlas N. Comprehensive molecular portraits of human breast tumours. Nature. 2012; 490:61–70.
21. Ringner M, Fredlund E, Hakkinen J, Borg A, Staaf J. GOBO: gene expression-based outcome for breast cancer online. PloS one. 2011; 6:e17911.
22. Gygi SP, Rochon Y, Franza BR, Aebersold R. Correlation between protein and mRNA abundance in yeast. Mol Cell Biol. 1999; 19:1720–1730.
23. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nature reviews Genetics. 2012; 13:227–232.
24. Li JJ, Bickel PJ, Biggin MD. System wide analyses have underestimated protein abundances and the importance of transcription in mammals. PeerJ. 2014; 2:e270.
25. Zhang B, Wang J, Wang X, Zhu J, Liu Q, Shi Z, Chambers MC, Zimmerman LJ, Shaddox KF, Kim S, Davies SR, Wang S, Wang P, Kinsinger CR, Rivers RC, Rodriguez H, et al. Proteogenomic characterization of human colon and rectal cancer. Nature. 2014; 513:382–387.
26. Weigelt B, Geyer FC, Reis-Filho JS. Histological types of breast cancer: how special are they? Molecular oncology. 2010; 4:192–208.
27. Metzger-Filho O, Tutt A, de Azambuja E, Saini KS, Viale G, Loi S, Bradbury I, Bliss JM, Azim HA, Jr., Ellis P, Di Leo A, Baselga J, Sotiriou C, Piccart-Gebhart M. Dissecting the heterogeneity of triple-negative breast cancer. J Clin Oncol. 2012; 30:1879–1887.
28. Duffy MJ, McGowan PM, Crown J. Targeted therapy for triple-negative breast cancer: where are we? Int J Cancer. 2012; 131:2471–2477.
29. Massa F, Devader C, Beraud-Dufour S, Brau F, Coppola T, Mazella J. Focal adhesion kinase dependent activation of the PI3 kinase pathway by the functional soluble form of neurotensin receptor-3 in HT29 cells. Int J Biochem Cell Biol. 2013; 45:952–959.
30. Massa F, Devader C, Lacas-Gervais S, Beraud-Dufour S, Coppola T, Mazella J. Impairement of HT29 Cancer Cells Cohesion by the Soluble Form of Neurotensin Receptor-3. Genes & cancer. 2014; 5:240–249.
31. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. Cell. 2011; 147:275–292.
32. Mitra SK, Schlaepfer DD. Integrin-regulated FAK-Src signaling in normal and cancer cells. Curr Opin Cell Biol. 2006; 18:516–523.
33. Lewin GR, Nykjaer A. Pro-neurotrophins, sortilin, and nociception. Eur J Neurosci. 2014; 39:363–374.
34. Andersen JL, Schroder TJ, Christensen S, Strandbygard D, Pallesen LT, Garcia-Alai MM, Lindberg S, Langgard M, Eskildsen JC, David L, Tagmose L, Simonsen KB, Maltas PJ, Ronn LC, de Jong IE, Malik IJ, et al. Identification of the first small-molecule ligand of the neuronal receptor sortilin and structure determination of the receptor-ligand

complex. Acta crystallographica Section, D, Biological crystallography. 2014; 70:451–460.

35. Longo FM, Massa SM. Small-molecule modulation of neurotrophin receptors: a strategy for the treatment of neurological disease. Nature reviews Drug discovery. 2013; 12:507–525.
36. Pundavela J, Demont Y, Jobling P, Lincz LF, Roselli S, Thorne RF, Bond D, Bradshaw RA, Walker MM, Hondermarck H. ProNGF Correlates with Gleason Score and Is a Potential Driver of Nerve Infiltration in Prostate Cancer. The American journal of pathology. 2014; 184:3156–3162.
37. Vanhecke E, Adriaenssens E, Verbeke S, Meignan S, Germain E, Berteaux N, Nurcombe V, Le Bourhis X, Hondermarck H. Brain-derived neurotrophic factor and neurotrophin-4/5 are expressed in breast cancer and can be targeted to inhibit tumor cell survival. Clin Cancer Res. 2011; 17:1741–1752.

A.2 LICENSE TO USE THE COPYRIGHT MATERIAL IN CHAPTER 3 OF THIS THESIS

ELSEVIER LICENSE TERMS AND CONDITIONS

Jan 14, 2015

This is a License Agreement between Jay Pundavela ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Jay Pundavela
Customer address	University Drive Callaghan, NSW 2291
License number	3547500730656
License date	Jan 14, 2015
Licensed content publisher	Elsevier
Licensed content publication	The American Journal of Pathology
Licensed content title	ProNGF Correlates with Gleason Score and Is a Potential Driver of Nerve Infiltration in Prostate Cancer
Licensed content author	Jay Pundavela,Yohann Demont,Phillip Jobling,Lisa F. Lincz,Severine Roselli,Rick F. Thorne,Danielle Bond,Ralph A. Bradshaw,Marjorie M. Walker,Hubert Hondermarck
Licensed content date	December 2014
Licensed content volume number	184
Licensed content issue number	12
Number of pages	7
Start Page	3156
End Page	3162
Type of Use	reuse in a thesis/dissertation
Portion	full article
Format	both print and electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Title of your thesis/dissertation	ProNGF/NGF and Nerve Infiltration in Prostate and Breast Cancer
Expected completion date	Feb 2015
Estimated size (number	

of pages)	
Elsevier VAT number	GB 494 6272 12
Price	0.00 AUD
VAT/Local Sales Tax	0.00 AUD / 0.00 GBP
Total	**0.00 AUD**

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.
3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."
4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.
5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)
6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.
10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.
12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).
13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.
14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the

contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

For journal authors: the following clauses are applicable in addition to the above: Permission granted is limited to the author accepted manuscript version* of your paper.

***Accepted Author Manuscript (AAM) Definition:** An accepted author manuscript (AAM) is the author's version of the manuscript of an article that has been accepted for publication and which may include any author-incorporated changes suggested through the processes of submission processing, peer review, and editor-author communications. AAMs do not include other publisher value-added contributions such as copy-editing, formatting, technical enhancements and (if relevant) pagination.

You are not allowed to download and post the published journal article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version. A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx. As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

Posting to a repository: Authors may post their AAM immediately to their employer's institutional repository for internal use only and may make their manuscript publically available after the journal-specific embargo period has ended.

Please also refer to Elsevier's Article Posting Policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only.. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

Elsevier Open Access Terms and Conditions

Elsevier publishes Open Access articles in both its Open Access journals and via its Open Access articles option in subscription journals.

Authors publishing in an Open Access journal or who choose to make their article Open Access in an Elsevier subscription journal select one of the following Creative Commons user licenses, which define how a reader may reuse their work: Creative Commons Attribution License (CC BY), Creative Commons Attribution – Non Commercial - ShareAlike (CC BY NC SA) and Creative Commons Attribution – Non Commercial – No Derivatives (CC BY NC ND)

Terms & Conditions applicable to all Elsevier Open Access articles:
Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation.
The author(s) must be appropriately credited.
If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.
Additional Terms & Conditions applicable to each Creative Commons user license:
CC BY: You may distribute and copy the article, create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), to include in a collective work (such as an anthology), to text or data mine the article, including for commercial purposes without permission from Elsevier
CC BY NC SA: For non-commercial purposes you may distribute and copy the article, create extracts, abstracts and other revised versions, adaptations or derivative works of or from an article (such as a translation), to include in a collective work (such as an anthology), to text and data mine the article and license new adaptations or creations under identical terms without permission from Elsevier
CC BY NC ND: For non-commercial purposes you may distribute and copy the article and include it in a collective work (such as an anthology), provided you do not alter or modify the article, without permission from Elsevier
Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.
Commercial reuse includes:

- Promotional purposes (advertising or marketing)
- Commercial exploitation (e.g. a product for sale or loan)
- Systematic distribution (for a fee or free of charge)

Please refer to Elsevier's Open Access Policy for further information.

21. **Other Conditions**: Permission is granted to submit your article in electronic format. This license permits you to post this Elsevier article online if it is embedded within your thesis. You are also permitted to post your Author Accepted Manuscript online; however posting of the final published article is prohibited. Please refer to Elsevier's Posting Policy for further information: http://www.elsevier.com/wps/find/authors.authors/postingpolicy

v1.7

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

A.3 LIST OF ABSTRACTS FROM CONFERENCES/SYPOSIUMS

Pundavela J, Roselli S, Choquet-Katylevsky G, Keene S, de Oliveira CS, Scott RJ, and Hondermarck H, ***Characterisation and validation of monoclonal antibodies against pro-nerve growth factor for investigating its potential as a biomarker and target in breast cancer metastasis***, 5th Annual Hunter Cancer Research Symposium, Newcastle, November 2012.

Pundavela J, Roselli, Choquet-Katylevsky G, Keene S, Scott RJ, and Hondermarck H, ***Development of a monoclonal antibody against pro-nerve growth factor for diagnostic and therapeutic purpose in breast cancer***, Translational Cancer Research Conference, Newcastle, NSW Australia, 23 – 25 October 2013.

Roselli S, Pundavela J, Choquet-Katylevsky G, Bradshaw RA, Scott RJ, and Hondermarck H, ***PronGF and its receptors as clinical biomarker and targets in breast cancer***, Translational Cancer Research Conference, Newcastle, NSW Australia, 23 – 25 October 2013.

Pundavela J, Demont Y, Lincz LF, Roselli S, Bond D, Walker MM, Hubert Hondermarck H, ***ProNGF correlates with Gleason score and is a potential driver of nerve infiltration in prostate cancer***, 7th Australian Health and Medical Research Congress, Melbourne, VIC Australia 2014

Pundavela J, Roselli S, Demont Y, Faulkner S, Attia J, Keene S, Walker MM, Hondermarck H, ***The neuronal protein sortilin is expressed in aggressive breast cancers and participates in tumor cell growth and invasion***, 37th Annual San Antonio Breast Cancer Symposium, San Antonio, TX USA 2014

Jobling P, Roselli S, Pundavela J, Faulkner S, Oliveira SMR, Demont Y, Walker MM, Bradshaw RA and Hondermarck H, ***Neuronal development in tumors: a novel promoter of cancer progression***, Hunter Meeting, NSW Australia 2015

A.4 LIST OF AWARDS

2014

MARGARET TAYLOR TRAVEL AWARD

HUNTER MEDICAL RESEARCH INSTITUTE

2012

BEST POSTER – BASIC/BIOMEDICAL SCIENCE

AWARDED BY LIFE TECHNOLOGIES

5TH ANNUAL HUNTER CANCER RESEARCH SYPOSIUM

HUNTER MEDICAL RESEARCH INSTITUTE

2012

AUSTRALIAN POSTGRADUATE AWARD

SCHOOL OF BIOMEDICAL SCIENCE AND PHARMACY

UNIVERSITY OF NEWCASTLE