ProNGF, NGF and their receptors in tumour innervation and progression: a study in breast and thyroid cancers.

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Declarations

TESTIMONY OF ORIGINALITY

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision. The thesis contains no material which has been accepted, or is being examined, 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 and any approved embargo.

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List of Publications Included as Part of this Thesis

Boilly B*, Faulkner S*, Jobling P, Hondermarck H: **Nerve Dependence: From Regeneration to Cancer**. *Cancer Cell*. 2017 Mar 13;31(3):342-354.

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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 Oncol.* 2015 Oct;9(8):1626-35.

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Faulkner S, Jobling P, Rowe C, Rodriguez-Oliveira S, Roselli S, Thorne R, Oldmeadow C, Attia J, Jiang CC, Zhang XD, Walker MM, Hondermarck H: **Neurotrophin Receptors TrkA, p75^{NTR} and Sortilin are Increased and Targetable in Thyroid Cancer**. *Am J Pathol.* 2018 Jan;188(1):229-241.

List of Abbreviations

0-9

6OHDA	6-Hydroxydopamine

A

ATCC	American Type Culture Collection
AA	Amino Acid
ANOVA	Analysis of Variance
ATC	Anaplastic Thyroid Carcinoma
AGR	Anterior Gradient Protein
AUROC	Area Under the Receiver-Operating Characteristic Curve

B

BMP	Bone Morphogenetic Protein
BDNF	Brain-Derived Neurotrophic Factor

C

CSC	Cancer Stem Cell
CNS	Central Nervous System
CI	Confidence Interval
CRD	Cysteine-Rich Domain

D

DD	Death Domain
DNA	Deoxyribonucleic Acid
DAB	3,3'-Diaminobenzidine
DRG	Dorsal Root Ganglia
DCIS	Ductal Carcinoma In Situ
DMEM	Dulbecco’s Modified Eagle Medium

E

EGF	Epidermal Growth Factor
ER	Estrogen Receptor

ECM	Extracellular Matrix
ERK	Extracellular Signal-Regulated Kinase

F

FGF	Fibroblast Growth Factor
FNAB	Fine Needle Aspiration Biopsy
FCS	Foetal Calf Serum
FTC	Follicular Thyroid Carcinoma

G

GDNF	Glia-Derived Neurotrophic Factor
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
G-CSF	Granulocyte-Colony Stimulating Factor

H

HER2	Human Epidermal Growth Factor Receptor 2
HME	Human Mammary Epithelium

I

IgG	Immunoglobulin G
IHC	Immunohistochemistry
IR-Dye	Infrared Dye
IDC	Invasive Ductal Carcinoma
ILC	Invasive Lobular Carcinoma

J

JNK	c-Jun N-terminal Kinase

K

kDa	Kilodalton

M

MTC	Medullary Thyroid Carcinoma

mRNA	Messenger Ribonucleic Acid
MAPK	Mitogen-Activated Protein Kinase
MW	Molecular Weight

N

NGF	Nerve Growth Factor
NGFR	Nerve Growth Factor Receptor
NT	Neurotrophin
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
NTRK1	Neurotrophin Tyrosine Receptor Kinase 1
nAG	Newt Anterior Gradient
NFkB	Nuclear Factor-κB

P

$p75^{NTR}$	p75 neurotrophin receptor
PTC	Papillary Thyroid Carcinoma
PNI	Perineural Invasion
PBS	Phosphate Buffered Saline
PI3K	Phosphoinositide-3-Kinase
PLCγ	Phosphoinositide Phospholipase C Gamma
PDGF	Platelet-Derived Growth Factor
PCR	Polymerase Chain Reaction
PTR	Post-Translational Modification
proNGF	Precursor of NGF
PR	Progesterone Receptor
PGP9.5	Protein Gene Product 9.5

Q

qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction

R

ROC	Receiver-Operating Characteristic
RTK	Receptor Tyrosine Kinase
RAC	Rho GTPase
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute Media

S

siRNA	Small/Short Interfering RNA
SHH	Sonic Hedgehog
SORT	Sortilin
SAS	Statistical Analysis System

T

TPA	Tetradecanoyl Phorbol Acetate
TCGA	The Cancer Genome Atlas
TMA	Tissue Microarray
Trk	Tropomyosin-Related Kinase
TNF	Tumour Necrosis Factor
TRAF	Tumour Necrosis Factor Receptor-Associated Factor

V

VPS10P	Vacuolar Protein Sorting 10 Protein

Table of Contents

Abstract

Infiltration of the tumour microenvironment by nerve fibres, termed cancer neurogenesis, is a relatively understudied feature of human carcinogenesis. Until only recently, perineural invasion (PNI), a process by which cancer cells surround and invade nerves, was thought to be the sole interaction between both tumoural and neuronal populations. PNI has traditionally been associated with clinically advanced tumours, in which it is thought to provide an alternate route for metastasis, generally resulting in a relatively poor prognosis for the patients. Recent studies however have demonstrated that denervation can supress tumour growth and metastasis, suggesting that there is separate interplay between both cancer and neuronal cells, extending far beyond that of PNI. However, what is yet to be fully elucidated in the literature is the molecular mechanism or mediators at play, responsible for facilitating this nerve-cancer cell crosstalk. What has been hypothesised, and since proven in a handful of human cancers, is that trophic factors are released by nerves and are capable of acting on cancer or other cells encompassing the tumour microenvironment. Conversely, cancer cells release neurotrophic factors that are capable of stimulating nerve infiltration or neurogenesis of the tumour. Neurotrophins and their receptors are one such family of neurotrophic factors that are emerging targets in oncology. More specifically, NGF and its precursor protein proNGF, along with their receptors, TrkA, $p75^{NTR}$ and sortilin, have already been implicated in several human cancers, including but not limited to that of the breast, skin (melanoma) and prostate. The overarching aim of this thesis was to develop a greater understanding of the emerging importance of both nerves and neurotrophic growth factors in influencing the growth and dissemination of human cancers. More specifically, this body of work aims to elucidate the extent and role of nerve infiltration within the tumour microenvironment of breast and thyroid cancers, as well as to determine any associations with the expression and function of NGF, proNGF and their receptors, TrkA, $p75^{NTR}$ and sortilin.

In a large cohort of primary breast tumours, we detected neural infiltration using the broad neuronal marker, PGP9.5. Invasive ductal carcinomas had a higher proportion of nerves as compared with that of invasive lobular carcinomas as well

as ductal carcinomas in situ. Additionally, the secretion of NGF was detected from a series of breast cancer cell lines, within their conditioned culture media. Co-culturing breast cancer cells with that of neuronal-like cells resulted in neurite outgrowth, which was ablated with the use of an NGF blocking-antibody, highlighting its potential role in stimulating breast cancer neurogenesis.

Following this, we looked to further clarify the expression and function of both nerves and neurotrophic growth factors in thyroid cancer. ProNGF expression was analysed by immunohistochemistry in two cohorts of cancer versus benign and normal thyroid tissues. Although innervation of thyroid cancers has not been previously reported, using the neuronal marker PGP9.5 we detected nerves in primary thyroid tumours. In both cohorts, proNGF was found to be overexpressed in thyroid cancer cells, as compared with both thyroid adenomas and normal thyroid tissue. We also demonstrated that proNGF is secreted by anaplastic thyroid cancer cell lines, highlighting its potential as a diagnostic biomarker, both histologically and within that of the blood. Next we looked to define the expression of TrkA, $p75^{NTR}$ and sortilin in thyroid cancer, as well as to determine if targeting these receptors reduced features of aggressiveness. TrkA was found to be more commonly expressed in tumours, where it was found to be associated with lymph node metastasis. In addition, nerves in the tumour microenvironment were positive for TrkA. $P75^{NTR}$ was overexpressed in anaplastic thyroid cancers compared to papillary and follicular subtypes whereas sortilin was overexpressed in all histological subtypes, as compared with adenomas and normal thyroid tissue. Targeting TrkA, $p75^{NTR}$ and sortilin *in vitro* using a pair of anaplastic thyroid cancer cell lines decreased cell survival and features of metastasis (migration and invasion), thus highlighting their potential are novel therapeutic targets in this devastating subtype of thyroid disease. Taken together, the work portrayed in this thesis has provided new evidence highlighting the importance of nerve infiltration in human carcinomas of the breast and thyroid, elucidated a role for NGF as a potential regulator of neurogenesis in the breast tumour microenvironment, as well as implicated NGF, its precursor proNGF and their receptors (TrkA, $p75^{NTR}$ and sortilin) as novel targets for therapeutic intervention.