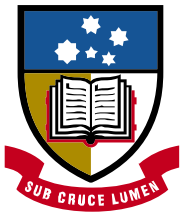

THE UNIVERSITY of ADELAIDE

A NEW ROLE FOR INFLAMMATORY PEROXIDASES IN BREAST CANCER DEVELOPMENT AND METASTASIS

Vasilios Panagopoulos (BBiotech)

A Thesis submitted for the degree of Doctor of Philosophy

in

The Discipline of Surgery, School of Medicine

Faculty of Health Science

The University of Adelaide

For my boys

Dion and Luka

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DECLARATION

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Vasilios Panagopoulos

ACKNOWLEDGEMENTS

"The highest reward for man's toil is not what he gets for it

but what he becomes by it."

John Ruskin

This quote resonates with me as striving to improve oneself, has more importance and value than any material reward. This thesis represents my '*toil'* and personifies my three-year journey filled with experiences as a PhD candidate, surrounded by truly remarkable people. These special few have not only shared this road with me, but have helped me become the best version of myself, the *'highest reward'*. It is an absolute necessity to have people like this in our lives to challenge us and raise our standards.

First and foremost I owe an immense amount of gratitude to my principle supervisor, Prof Andreas Evdokiou for giving me the opportunity to undertake a PhD project within the Breast Cancer Research Unit. Prof Evdokiou has not only been an exceptional mentor, but throughout my PhD has taken the role of a father, a brother, and most of important of all, a friend. I am ever grateful for your support and guidance throughout my PhD and you are someone whom I will always endeavour to emulate in the future.

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Next I would like to thank my family, my wife Romana together with my boys Dion and Luka. Who of which, are the sole reason that I aspire to be successful. None of this would be possible without their continual love, support, and patience. Everything I do is for them. Some people have told me how hard it must have been, and that they couldn’t imagine doing a PhD with a family. I disagree; I think nothing gives more motivation, greater self-worth, and satisfaction striving towards a better future for your family. Romana, you have given me nothing but love and understanding during the last 3 years, you are my neck. You will always hold me up and point me in the right direction. I will continue to be a dedicated husband and father and only hope our boys can look up to me with a sense of honour and pride, and within their eyes that I have been the best role model a father can be.

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ABSTRACT

Breast cancer is the leading cause of cancer mortality in women worldwide. Although we have made significant improvements in the detection and treatment of localized breast cancer with women surviving longer than ever before, patients with metastatic disease have a far worse prognosis. Metastasis is the leading cause of death in breast cancer patients with a 5-year survival of less than 30% and is currently without a cure. Thus, improvements in the development of new treatments will have profound impact on outcomes for these patients.

Recent insight into the interactions of stromal cells and cancer cells within the breast tumour microenvironment, such as immune cells, fibroblasts, extracellular matrix (ECM) proteins, and vasculature, have revealed that the stroma plays a critical role in regulating a favourable environment for breast cancer initiation, progression and metastasis. Therefore, aspects of stromal biology are the key focus for future studies to improve patient outcome, by identifying new targets for therapy development. The role of the innate immune infiltrates in regulating cancer progression has been receiving considerable attention over recent years. Clinical studies indicate the extent of neutrophil and eosinophil accumulation within the developing tumour microenvironment is strongly correlated with a poor patient prognosis and survival. Having infiltrated the tumour, these inflammatory cells deposit myeloperoxidase (MPO) and eosinophil peroxidase (EPO) in abundance. These enzymes have mainly been studied in the context of providing oxidative defence against invading pathogenic microorganisms, and to-date the functional consequence of their heightened presence within the tumour microenvironment has long been unclear.

The work in this thesis aims to provide a mechanistic link between inflammatory peroxidase enzymes MPO and EPO, influencing stromal cells within the tumour microenvironment leading to tumour development and metastasis. Using physiological

relevant ranges of peroxidases this study revealed that peroxidases possess a well-conserved pro-fibrogenic capacity to stimulate the migration of fibroblastic cells and promote their ability to secrete collagenous proteins to generate a functional ECM using both *in vitro* and *in vivo* approaches. Structurally, the ECM generated upon peroxidase stimulation demonstrated highly linearized collagen fibers synonymous with tumour ECM that is highly conducive for cancer cell adhesion, and invasion. This suggests that peroxidase-mediated collagen biosynthesis by cancer-associated fibroblasts may play an important role in tumour progression.

The stimulation of angiogenesis is considered to be one of the most important hallmarks of tumour progression. Data in this thesis demonstrates for the first time the pro-angiogenic capacity of both MPO and EPO to significantly enhance the *in vitro* proliferation, migration and capillary formation of human endothelial cells that are fundamental in the process of angiogenesis. The use of a murine model of angiogenesis confirmed the ability of these peroxidase enzymes to facilitate the generation of functional blood vessels in an *in vivo* model. These results suggest that MPO and EPO play a crucial role as drivers of angiogenesis, with the potential to support the vascularization of tumours and promote growth and metastasis. In addition, the pro-angiogenic and pro-fibrotic activity displayed by peroxidases was blocked using the peroxidase inhibitor 4-ABAH, indicating that the catalytic activity is essential for its activity.

Using the orthotopic 4T1 mouse mammary carcinoma model, we demonstrated for the first time that delivery of MPO and EPO directly into developing tumours increased primary tumour burden and concomitant with enhanced lung metastases. Histological inspection confirmed that MPO and EPO stimulated an increase in endothelial cell recruitment, fibroblast activation and collagen deposition within these tumours.

In conclusion, our findings demonstrate for the first time that the peroxidase enzymes MPO and EPO confer a broader range of action than previously thought and exhibit potent effects in the tumour milieu on matrix function, composition, angiogenesis, tumour invasion and metastasis. Importantly, these studies identify the use of peroxidase inhibitors that block the catalytic activity of the enzyme, as a potential novel therapeutic strategy for breast cancer prevention and therapy.

PUBLICATIONS

Summary: Published Journal Articles: **8**; Submitted Journal Articles **3**; Paper in preparation **3**; Patent Applications **1**.

Peer Reviewed Journal Articles:

1. Leach, DA., **Panagopoulos, V**., Nash, C., Thomson, AA., Selth, LA., and Buchanan, G., (2016) Cell-lineage specificity and role of AP-1 in the prostate fibroblast androgen receptor cistrome. *Mol Cell Endocrinol,* 384**,** 185-99.

2. DeNichilo, MO., Shoubridge, AJ., **Panagopoulos, V**., Liapis, V., Zinonos, I., Hay, S., Atkins, GJ., Findlay, DM., and Evdokiou, A., (2016). Peroxidase enzymes regulate collagen biosynthesis and matrix mineralization by cultured human osteoblasts. *Calcif Tissue Int. 98(3):294-305*

3. **Panagopoulos, V**., Zinonos, I., Leach, D. A., Hay, S. J., Liapis, V., Zysk, A., Ingman, W. V., DeNichilo, M. O. and Evdokiou, A. (2015). Uncovering a new role for peroxidase enzymes as drivers of angiogenesis. *Int J Biochem Cell Biol, 68, 128-138.*

4. DeNichilo, M. O., **Panagopoulos, V**., Rayner, T. E., Borowicz, R. A., Greenwood, J. E. and Evdokiou, A. (2015). Peroxidase enzymes regulate collagen extracellular matrix biosynthesis. *Am J Pathol. 185, 1372-1384.*

5. Liapis, V., Labrinidis, A., Zinonos, I., Hay, S., Ponomarev, V., **Panagopoulos, V**., DeNichilo, MO., Ingman, W., Atkins, GJ., Findlay, DM., Zannettino, AC., and Evdokiou, A., (2015). Hypoxia-activated pro-drug TH-302 exhibits potent tumor

suppressive activity and cooperates with chemotherapy against osteosarcoma. *Cancer Lett 357(1):160-169.*

6. Liapis, V., Zinonos, I., Hay, S., Ponomarev, V., **Panagopoulos, V**., DeNichilo, MO., Ingman, W., Atkins, GJ., Findlay, DM., Zannettino, AC., and Evdokiou, A., (2015). Anticancer efficacy of the hypoxia-activated prodrug evofosfamide (TH-302) in osteolytic breast cancer murine models. *Cancer Med 5(3):534-45.*

7. Zinonos, I., Labrinidis, A., Liapis, V., Hay, S., **Panagopoulos, V**., DeNichilo, MO., Ponomarev, V., Ingman, W., Atkins, G.J, Findlay, DM., Zannettino, AC., and Evdokiou, A., (2014). Doxorubicin overcomes resistance to drozitumab by antagonizing Inhibitor of Apoptosis Proteins (IAPs). *Anticancer Res 34(12):7007-7020.*

8. Zinonos, I., Luo, KW., Labrinidis, A., Liapis, V., Hay, S., **Panagopoulos, V**., DeNichilo, MO., Ko, CH., Yue, GG., Lau, CB, Ingman, W., Ponomarev, V., Atkins, GJ., Findlay, DM., Zannettino, AC., and Evdokiou, A., (2014). Pharmacologic inhibition of bone resorption prevents cancer-induced osteolysis but enhances soft tissue metastasis in a mouse model of osteolytic breast cancer. *Int J Oncol 45(2):532-540.*

Submitted Articles:

1. **Panagopoulos, V**., Leach, DA., Zinonos, I., Ponomarev, V., Licari, G., Liapis, V., Ingman, W., Anderson, P., DeNichilo, MO., and Evdokiou, A., (2016). Inflammatory Peroxidases Promote Breast Cancer Progression in Mice via Regulation of the Tumour Microenvironment (Submitted to *International Journal of Oncology*).

2. **Panagopoulos, V.**, Liapis, V., Zinonos, I., Hay, S., Leach, DA., Ingman, W., DeNichilo, MO., Atkins G.J., Findlay D.M., Zannettino A.C.W., and Evdokiou, A., (2016). Peroxidase enzymes inhibit osteoclast differentiation and bone resorption (Submitted to *Molecular and Cellular Endocrinology*).

3. Wang Y., Zinonos I., Zysk A., **Panagopoulos V**., Kaur G., Santos A., Losic D., and Evdokiou A., (2016) *In vivo* Toxicological Assessment of Electrochemically Engineered Anodic Alumina Nanotubes: A Study of Biodistribution, Subcutaneous Implantation and Intravenous Injection (Submitted to the journal of *Advanced Healthcare Materials*).

Articles in Progress:

1. Liapis V., Zysk A., DeNichilo M., Zinonos I., Hay S., **Panagopoulos V**., Shoubridge A., Difelice C., Ponomarev V., Ingman W., Atkins GJ., Findlay DM., Zannettino ACW., and Evdokiou A., (2016). Anticancer efficacy of the hypoxia activated prodrug evofosfamide is enhanced in combination with proapoptotic receptor agonists, dulanermin and drozitumab against osteosarcoma.

2. **Panagopoulos, V.**, Zinonos, I., Grubor-Bauk, B., Leach DA., DeNichilo, MO, and Evdokiou, A., (2016). Peroxidase Enzymes inhibit immune cell-mediated killing of breast cancer cells *in vitro*.

3. Leach, DA., **Panagopoulos, V.**, Trotta, AP., Need, EF., and Buchanan, G., (2016). Androgen receptor regulation of fibroblast secreted ECM mediates prostate cancer invasion and patient outcomes by altering ECM microarchitecture.

Patent Application:

1. Evdokiou, A.; DeNichilo, MO.; **Panagopoulos, V.** Modulation of Angiogenesis or Osteogenesis by Modulation Peroxidase Functionality. A Provisional patent application filed on 25 June 2014. *Australian Patent number AU2014902437.*

CONFERENCE PRESENTATIONS

- **Vasilios Panagopoulos,** Damien A Leach, Irene Zinonos, Shelley Hay, Giovanni Licari, Vasilios Liapis, Wendy Ingman, Vladimir Ponomarev, Peter Anderson, Mark O DeNichilo and Andreas Evdokiou. Peroxidases Promote Breast Cancer Development and Metastasis in Mice via Regulation of ECM Properties and Angiogenesis. ASMR SA Annual Scientific Meeting 2016, Adelaide, Australia. *Oral presentation.*

- **Vasilios Panagopoulos,** Irene Zinonos, Damien A Leach, Shelley Hay, Vasilios Liapis, Wendy Ingman, Jenny Hardingham, Vladimir Ponomarev, Mark O DeNichilo and Andreas Evdokiou. A new role for inflammatory peroxidases in cancer development and metastasis. 54th ASMR National Scientific Conference 2015, Adelaide, Australia. *Poster presentation.*

- **Vasilios Panagopoulos,** Irene Zinonos, Damien A Leach, Shelley Hay, Vasilios Liapis, Aneta Zysk, Wendy Ingman, Vladimir Ponomarev, Mark O DeNichilo and Andreas Evdokiou. A new role for inflammatory peroxidases in breast cancer development and metastasis. The Queen Elizabeth Hospital (TQEH) Research Day 2015, Adelaide Australia. *Oral presentation.*

- **Vasilios Panagopoulos,** Irene Zinonos, Damien A Leach, Shelley Hay, Vasilios Liapis, Wendy Ingman, Vladimir Ponomarev, Mark O DeNichilo and Andreas Evdokiou. A new role for inflammatory peroxidases in cancer development and metastasis. ASMR SA Annual Scientific Meeting 2015, Adelaide, Australia. *Oral presentation.*

- **Vasilios Panagopoulos,** Irene Zinonos, Damien A Leach, Shelley Hay, Vasilios Liapis, Aneta Zysk, Wendy Ingman, Vladimir Ponomarev, Mark O DeNichilo and Andreas Evdokiou. A new role for inflammatory peroxidases in breast cancer development and metastasis. 9th Annual Florey International Postgraduate Research Conference 2015, Adelaide, Australia. *Poster presentation*.

- **Vasilios Panagopoulos,** Irene Zinonos, Damien A Leach, Shelley Hay, Vasilios Liapis, Aneta Zysk, Wendy Ingman, Vladimir Ponomarev, Mark O DeNichilo and Andreas Evdokiou. Uncovering a new role for peroxidases in breast cancer development and metastasis. OzMRS-CTx Metastasis Workshop 2014, Melbourne, Australia. *Oral and poster presentation.*

- **Vasilios Panagopoulos,** Irene Zinonos, Damien A Leach, Shelley Hay, Vasilios Liapis, Aneta Zysk, Wendy Ingman, Vladimir Ponomarev, Mark O DeNichilo and Andreas Evdokiou. Uncovering a new role for peroxidases in breast cancer development, progression and metastasis. The Queen Elizabeth Hospital (TQEH) Research Day 2014, Adelaide Australia. *Oral presentation.*

- **Vasilios Panagopoulos,** Irene Zinonos, Damien A Leach, Shelley Hay, Vasilios Liapis, Aneta Zysk, Wendy Ingman, Vladimir Ponomarev, Mark O DeNichilo and Andreas Evdokiou. Uncovering a new role for peroxidases in breast cancer development and metastasis. Florey International Postgraduate Research Conference 2014, Adelaide, Australia. *Poster presentation*.

- **Vasilios Panagopoulos,** Irene Zinonos, Damien A Leach, Shelley Hay, Vasilios Liapis, Aneta Zysk, Wendy Ingman, Vladimir Ponomarev, Mark O DeNichilo and Andreas Evdokiou. Uncovering a New Role for Peroxidases in Breast Cancer Development and Metastasis. Metastasis Research Society (MRS) Congress 2014, Heidelberg, Germany. *Oral and poster presentation.*

PRIZES AWARDED

- Best Poster Presentation for the Florey Medical Research Foundation Prize, 2015. 9th Annual Florey International Postgraduate Research Conference, Faculty of Health Sciences, University of Adelaide, 24 September 2015, National Wine Centre of Australia.

- Best Oral Presentation in the category for Senior PhD Students (Laboratory), 2014. TQEH Research Day, The Queen Elizabeth Hospital, 17 October 2014, Basil Hetzel Institute.

- Best Poster Presentation for the Florey Medical Research Foundation Prize, 2014. 8th Annual Florey International Postgraduate Research Conference, Faculty of Health Sciences, University of Adelaide, 25 September 2014, National Wine Centre of Australia.

- Best Poster Presentation for the School of Medicine Prize, 2014. 8th Annual Florey International Postgraduate Research Conference, Faculty of Health Sciences, University of Adelaide, 25 September 2015, National Wine Centre of Australia.

- Faculty of Health Sciences divisional PhD Scholarship, University of Adelaide.

ABBREVIATIONS

3D	3 Dimensional
4-ABAH	4-Amino Benzoic Acid Hydrazide
α-SMA	alpha-Smooth Muscle Actin
AA	Ascorbic Acid/Ascorbate
Akt	v-Akt murine Thymoma viral oncogene homolog 1
ANOVA	Analysis of Variance
BALB/C	Bagg albino (inbred mouse strain)
BLI	Bioluminescence Imaging
BM	Basement Membrane
BRCA	Breast Cancer mutation
BSA	Bovine Serum Albumin
CAF	Cancer Associated Fibroblast
cDNA	Complementary Deoxyribonucleic Acid
COX-2	Cyclooxygenase-2

DAB	3,3’-diaminobenzidine
DAPI	4’,6-diamidino-2-phenylindole
DCIS	Ductal Carcinoma in situ
DFS	Disease Free Survival
DMEM	Dulbecco’s Modified Eagle Medium
DMOG	Dimethyloxaloylglycine
DNA	Deoxyribonucleic Acid
DRT	Dermal Regeneration Template
EBM	Endothelial Base Media
ECF	Enhanced Cyan Fluorescence
ECL	Enhanced Chemi-Luminescence
ECM	Extracellular Matrix
EDTA	Ethylene-diamine-tetra Acetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay
EMT	Epithelial-Mesenchymal Transition
EPC	Endothelial Progenitor Cell
EPO	Eosinophil Peroxidase

ER	Endoplasmic Reticulum
ERK	Extracellular Regulated Kinase
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
H&E	Hematoxylin and Eosin
H_2O_2	Hydrogen Peroxide
Hb	Hemoglobin
HC	High Concentration
HFF	Human Foreskin Fibroblast
HGF	Hepatocyte Growth Factor
HIF	Hypoxia Inducible Factor
HRP	Horseradish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cell

i.p.	Intra-peritoneal
IFN	Interferon
IgG	Immunoglobulin-G
IL	Interleukin
JNK	Jun N-terminal kinase
LCIS	Lobule Carcinoma in situ
LOX	Lysyl Oxidase
LOXL	Lysyl Oxidase Like
LPO	Lactoperoxidase
LSGS	Low Serum Growth Supplement
M-CSF	Macrophage Colony-Stimulating Factor
MAPK	Mitogen Activated Protein Kinase
MCP	Monocyte Chemoattractant protein
MET	Mesenchymal-epithelial transition
MMP	Matrix Metalloproteinase
MPO	Myeloperoxidase

mRNA	Messenger Ribonucleic Acid
RNA	Ribonucleic Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
P4H	Prolyl-4 Hydroxylase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline-Tween
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PDGFRα	Platelet-Derived Growth Factor Receptor alpha
PET	Polyethylene Terephthalate
PF	Platelet Factor
PLOD	Procollagen-lysine 2-oxyglutarate 5-dioxygenase
PVDF	Polyvinylidene Fluoride
qPCR	Quantitative Polymerase Chain Reaction
RANK	Receptor activator of nuclear factor kappa-B receptor

RANKL	Receptor activator of nuclear factor kappa-B ligand
RNA	Ribonucelic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
RT-PCR	Real Time Polymerase Chain Reaction
SBP	Soybean Peroxidase
SDF-1	Stromal Cell Derived Factor 1
SEM	Standard Error of the Mean
TBST	Tris Buffered Saline Tween
TGF-β	Transforming Growth Factor beta
TNF-α	Tumor Necrosis Factor alpha
TRAP	Tartrate-resistant acid phosphatase
VEGF	Vascular Endothelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor Receptor 2

WBC	White Blood Cell
WGFE	Whey Growth Factor Extract
WT	Wild Type
ZEB1	Zinc Finger E-Box Binding Homeobox 1