THE UNIVERSITY OF HULL

Investigating the Biological and Clinical Implications of Endo180 in Breast Cancer

being a Thesis submitted for the Degree of Doctor of Philosophy

in the University of Hull

by

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June 2017

Abstract

Endo180 is a 180 kDa type-1 transmembrane receptor that belongs to the mannose receptor family and interacts with extracellular / intracellular collagens. Endo180 expression is correlated with tumour grade, disease progression and poor prognosis in several cancer types including breast cancer and prostate cancer, and Endo180-dependent collagen remodelling is proved to have mechanistic roles in the promotion of breast cancer progression and metastatic bone lesion pathology. It has recently been reported that Endo180 ectodomain is released from breast cancer cells and that measurement of Endo180 in plasma – alone or in combination with CA 15-3 antigen – provides an accurate method for classifying metastatic bone disease. It is also indicated that Endo180 levels in plasma are modulated following treatment with bisphosphonates. Therefore, the aim of this project is to further explore the clinical and biological implications of Endo180 in breast cancer. Prior to that, an enzyme-linked immunosorbent assay (ELISA) for Endo180 quantification was first developed and optimised, to facilitate further consideration of Endo180 in the broader clinical setting. The newly developed assay provided an accurate and efficient method for Endo180 measurement in different types of samples. Following the development of ELISA, Endo180 levels in prospectively collected patient samples were measured, and retrospective-blinded-evaluation was conducted in collaboration with the University of Liverpool. The result indicated that the level of plasma Endo180 in advanced breast cancer patients were significantly higher than early breast cancer patients. To investigate Endo180 modulation under bisphosphonate treatment, *in vitro* treatment of breast/prostate cancer cells and osteoblasts with bisphosphonates were carried out. The result showed that the expression of Endo180 in breast cancer cell MDA-MB-231 was inhibited by bisphosphonates, which led to a decrease of Endo180 levels on the cell

surface and released into the media. On the contrary, Endo180 expression and release levels in osteoblasts were not significantly influenced by bisphosphonates, with an increase on cell surface Endo180 level under the treatment. To further explore the mechanism under the release of Endo180, several factors that might be functional in the release of Endo180 from tumour cells and osteoblasts were investigated. It was found that MMPs could participate in the release of Endo180 from breast cancer cells, and MMP-2 was shown to influence the level of Endo180 that forms complex with other molecules. We also found that enabling Endo180 expression in MCF-7 cells could trigger the activities of MMP-2 / -9. The indirect co-culture of breast cancer cells and osteoblasts did not influence the release of Endo180 from breast cancer cells, while osteoblasts showed a decrease in Endo180 release. Indirect co-culture with osteoblasts could trigger the intake of Endo180 by prostate cancer cells, while osteoblasts were not influenced. From this study, we have shown the feasibility of plasma Endo180 as a potential biomarker for advanced breast cancer, the regulation of Endo180 release and expression by bisphosphonates, and the potential mechanisms for Endo180 release.

Key words: Advanced breast cancer; metastasis; Endo180; tumour marker; MMP; bisphosphonates; breast cancer cells; osteoblasts; co-culture; ELISA

Acknowledgements

Foremost, I would like to express my sincere gratitude to my supervisor, Dr Justin Sturge, for the continuous support of my PhD study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this project. I would also like to thank my secondary supervisor, Dr Anne-Marie Seymour, for her guidance and support.

I want to thank Prof Carlo Palmieri and his research team at the University of Liverpool, for the collaborations in the clinical study of this project.

My sincere thanks also go to all principal investigators of the cancer research group at the University of Hull: Dr Camille Ettelaie, Dr Chris Cawthorne, Dr Elena Rosca, Dr Graeme Stasiuk, Dr Isabel Pires, Dr Jenny Waby, Prof John Greenman, Dr Lynn Cawkewell, Dr Stephen Maher, for the guidance, encouragement and insightful advices they provided. I also want to thank our laboratory technicians Cordula Kemp, Ellie Beeby, Emma Oldridge, Linsey Malcolm and Sage Pickwell for their assistance.

I would also like to thank my fellow colleagues: Anna Todd, Becky Bibby, Brittany Wingham, Cecilia Miranda, Flore-Anne Poujade, Fuli Zheng, Hannah Moody, James Thompson, Jianlong Li, Kamil Talar, Nishadh Wijesinghe and Shubhanchi Nigam, for the inspiring discussions and encouragements at work.

I express thanks to China Scholarship Council and the University of Hull, for providing me with the opportunity to undertake this project, to attend academic conferences and to meet with researchers from all around the world.

Finally, special thanks to my family and friends, with whom I share my joy and sorrow. Especially to Lu Wang, for her consistent support, understanding and accompany.

Statement of original authorship

The work contained within this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

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Abbreviations

3D	Three-dimensional	**EGF**	Epidermal growth factor
ADAM	A disintegrin and metalloproteinase	**ELISA**	Enzyme-linked immmunosorbent assay
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs	**EMMPRIN**	Extracellular matrix metalloproteinase inducer
APS	Ammonium persulfate	**EMT**	Epithelial – mesenchymal transition
ATP	Adenosine triphosphate	**Endo180**	Endocytic receptor 180
BALP	Bone alkaline phosphatase	**ER**	Estrogen receptor
BCA	Bicinchoninic acid	**FACS**	Fluorescence-activated cell sorting
BCS	Breast-conserving surgery	**FBS**	Fetal bovine serum
BM	Basement membrane	**FDA**	United States Food and Drug Administration
BMM	Bone marrow derived macrophage	**FGF**	Fibroblast growth factor
BMSC	Bone marrow derived stromal cell	**FNII**	Fibronectin type II-like domain
BSA	Bovine serum albumin	**FOXC2**	Forkhead box protein 2
CA	Carcinoma antigen	**GEF**	Guanine nucleotide exchange factor
Cadherin	Ca^{2+} dependent adhesion molecule family	**GSC**	Homeobox protein goosecoid
CD280	Cluster of differentiation 280	**GTP**	Guanosine triphosphate
CEA	Carcinoembryonic antigen	**HB-EGF**	Heparin-binding EGF-like growth factor
CRD	Cysteine-rich domain	**HER2**	Human epidermal growth factor receptor-2
CTLD	C-type lectin domain	**HGF**	Hepatocyte growth factor
CTX	carboxy-terminal cross-linking telopeptide of type I collagen	**HIF**	Hypoxia-inducible factor
DCIS	Ductal carcinoma in situ	**HMG-CoA**	3-hydroxy-3-methylglutaryl-coenzyme A
DMEC	Dermal microvascular endothelial cell	**hOB**	Human osteoblasts
DMEM	Dulbecco's modified Eagle's medium	**HRP**	Horseradish peroxidase
DMSO	Dimethyl sulfoxide	**HSC**	Hepatic stellate cell
E47	Transcription factor 3	**HUVEC**	Human umbilical vein endothelial cell
ECL	Electrochemiluminescence	**IFN-γ**	Interferon-γ
ECM	Extracellular matrix	**IGF**	Insulin-like growth factor
EDTA	Ethylenediaminetetraacetic acid	**IL-10**	Interleukin-10

KGF	Keratinocyte growth factor	**PVDF**	Polyvinylidene fluoride
LCIS	Lobular carcinoma in situ	**PyVT**	Polyomavirus middle T antigen
LOX	Lysyl oxidase	**RANK**	Receptor activator of NF-κB
LOX-L2	Lysyl oxidase-like 2	**RANKL**	Receptor activator of NF-κB ligand
mAb	Monoclonal antibody	**RIPA**	Radioimmunoprecipitation assay
MAPK	Mitogen-activated protein kinase	**ROCK**	Rho-associated protein kinase
MEF	Murine embryonic fibroblasts	**RT-PCR**	Reverse transcription polymerase chain reaction
MEM	Minimum essential medium	**SDS**	Sodium dodecyl sulfate
MET	Mesenchymal – epithelial transition	**SIM2**	Singleminded-2 transcription factor
miRNA	MicroRNA	**Smad**	Mothers against decapentaplegic protein
MLC	Myosin light chain	**Snail**	Snail family zinc finger protein
MMP	Matrix metalloproteinase	**SRE**	Skeletal-related events
MMTV	Mouse mammary tumour virus	**TBS**	Tris-buffered saline
MRC2	Macrophage mannose receptor, C-type 2	**TCA**	Trichloroacetic acid
N-BPs	Nitrogenous-containing bisphosphonates	**TEMED**	Tetramethylethylenediamine
NEAA	Non-essential amino acids	**TGF-β**	Transforming growth factor-beta
NF-κB	Nuclear factor-kappaB	**TIMP**	Tissue inhibitor of metalloproteinase
NICE	The National Institute for Health and Care Excellence	**TNM**	Tumour–node–metastasis
NP-40	Nonyl phenoxypolyethoxylethanol 40	**TUNEL**	Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling
OPG	Osteoprotegerin	**Twist**	Twist family basic helix-loop-helix protein
pAb	Polyclonal antibody	**uPA**	Urokinase-type plasminogen activator
PAGE	Polyacrylamide gel electrophoresis	**uPAR**	Urokinase-type plasminogen activator receptor
PBS	Phosphate-buffered saline	**uPARAP**	Urokinase-type plasminogen activator receptor associated protein
PDGF	Platelet-derived growth factor	**WNT**	Wingless-type MMTV integration site family protein
PFA	Paraformaldehyde	**WNT7B**	WNT family member 7B
PLA2R	Phospholipase A2 receptor	**Zeb**	Zinc finger E-box-binding homeobox
PSA	Prostate-specific antigen		

1 Introduction

In comparison to *in situ* carcinoma, advanced cancer, in which tumour cells have spread to secondary site(s), is much more difficult to be cured and significantly reduces the overall survival of cancer patients (Steeg, 2016). Metastatic disease is predicted to develop in one third of early breast cancer patients, and over 70% of advanced breast cancer cases will involve its dissemination to bone (Peto et al., 2012). The occurrence of bone metastasis is accompanied by the dysregulation of the homeostasis that normally exists between osteoblasts-mediated bone formation and osteoclasts-mediated bone degradation (Roodman 2004). In advanced breast cancer, the net result of the disruption to normal bone turnover is an increase in osteolysis and a subsequent reduction in bone quality, bone mass and bone strength (Mundy, 2002). The use of biomarkers in breast cancer patients is a valuable way to monitor the disease remission and progression, as well as the effectiveness of therapy (van Dalen et al., 1996, Robertson et al., 1999, Kurebayashi et al., 2004). The currently used biomarker for advanced breast cancer, CA 15-3, is not recommended to be used alone, instead, it should be measured in conjunction with diagnostic imaging and clinical examination (Harris et al., 2007, Cardoso et al., 2012). Therefore, the identification of a new sensitive biomarker for advanced breast cancer that can be used alone or in combination with other diagnostic tools could help improve the limitation of conventional biomarkers for advanced breast cancer.

One promising biomarker is collagen receptor Endo180, which is expressed by bone-forming osteoblasts and is required for normal bone homeostasis (Isacke et al., 1990, Engelholm et al., 2003). Endo180 is also expressed in breast tumour cells and can promote tumour growth (Wienke et al., 2007). Moreover, a soluble form of the

Endo180 ectodomain can be released from breast tumour cells, the level of which in the plasma of breast cancer patients is proved to be correlated with metastases that affects viscera and bone, and is modulated by bisphosphonate treatment (Palmieri et al., 2013a). Therefore, the main aim of this project is to further explore the potential of Endo180 being used as an accurate biomarker for advanced breast cancer, and to investigate the mechanisms underlying its release. The detailed research background for this project is discussed in Chapter 2, followed by materials and methods in Chapter 3.

In previous studies, protein immunoblot was used to analyse the level of Endo180 in conditioned medium and patient plasma samples (Palmieri et al., 2013a). As a semi quantitative analytical method, it is difficult to measure the absolute concentrations of the target protein using protein immunoblot. This technical limitation has hindered Endo180 measurement to be applied in a broader clinical setting. Hence, the initial focus of this project was to develop a more accurate and high-throughput quantitative assay, to facilitate the validation and clinical application of Endo180 measurement as a diagnostic tool. In this project, an ELISA for Endo180 quantification was established and validated. The development and validation of Endo180 ELISA is described in Chapter 4.

The pivotal study that forms the basis of this project by Palmieri et al. (2013) concluded that Endo180 is a potential biomarker for metastatic breast cancer, as plasma Endo180 levels were significantly higher in recurrent/metastatic breast cancer patients in comparison to early/localised breast cancer patients. With the newly established Endo180 ELISA, we carried out further analyses on plasma samples that were collected from breast cancer patients by our co-investigators at the University of Liverpool. As can be seen from the results shown in Chapter 5, the level of plasma Endo180 in

advanced breast cancer patients is significantly higher than early breast cancer patients, which makes Endo180 a potential biomarker for advanced breast cancer. Further investigation will be followed up by the evaluation of Endo180 to confirm whether it can predict the response of recurrent locoregional breast cancer to bisphosphonate treatment.

Treatment of metastatic bone disease with bisphosphonates and other bone-resorption inhibitors can help reduce its associated skeletal related events, such as intractable pain, pathological fracture and spinal cord compression (Honore et al., 2000). This kind of palliative treatment mostly alleviates bone pain and improves the quality of life of cancer patients, with potential improvements in disease regression and morbidity outcomes such as overall survival (Cardoso et al., 2009). Moreover, bisphosphonates have been proved be beneficial for postmenopausal early breast cancer patients in reducing the rate of bone metastasis, and is recommended for all patients with high risk of skeletal fracture routine clinical practice to prevent cancer treatment-induced bone loss (Early Breast Cancer Trialists' Collaborative Group, 2015, Hadji et al., 2016). The correlation between the plasma Endo180 level and bisphosphonate treatment observed in advanced breast cancer patients implies that the regulation of Endo180 in tumour or bone cells could be one of the anti-tumour effects of bisphosphonates, and the release level of Endo180 has a potential to be used as an indicator for the treatment effect of bisphosphonates (Palmieri et al., 2013a). In order to investigate of how bisphosphonate treatment affects the expression and release of Endo180 in the context of bone metastasis, *in vitro* experiments were set up to treat breast/prostate cancer cells and osteoblasts with two widely used bisphosphonates, alendronic acid and zoledronic acid, followed by the analysis of Endo180 expression and release. The results shown in Chapter 6 indicate that bisphosphonates have different regulatory effects on Endo180

in tumour cells and osteoblasts. In tumour cells, bisphosphonates inhibit the expression of Endo180, as well as its release/shedding and levels on the cell surface. Bisphosphonates do not influence the expression of Endo180 in osteoblasts, but promoted the release of Endo180 from the cell surface.

The final stage of this project was to ascertain the mechanism(s) associated with the release of Endo180, which is described in Chapter 7. Given that a cleavage site of MMP-2 was predicted in the trans-membrane region of Endo180 protein (see Section □), the correlation between MMP-2 and Endo180 was first explored. The result of *in vitro* MMP-2 treatment indicates that MMP-2 dose not influence the release of Endo180 from breast cancer cells. However, it could mediate the disruption of the complex formed between Endo180 and other molecules, as the level of non-complexed (single molecular) Endo180 protein was increased by MMP-2 treatment while the total level remained the same (Section 7.3.1.1). Furthermore, Endo180-CD147 complex is essential for maintaining the epithelial phenotype of prostate epithelial cells (Rodriguez-Teja et al., 2015a). Moreover, the significant decrease of Endo180 release and increase of Endo180 on cell surface in breast cancer cells treated with MMP inhibitor suggests the potential role of other subtypes of MMP in Endo180 release. Beside the effect of MMPs on Endo180, it was observed that enabling the expression of Endo180 can trigger MMP-2 / -9 activities in breast cancer cells.

It has been demonstrated that Endo180-related function is dysregulated at the tumour-bone stromal interface due to its downregulation in osteoblasts and upregulation on tumour cells adjacent to each other (Caley et al., 2012). To investigate if the release of Endo180 is also dysregulated by tumour-bone interaction, an *in vitro* indirect co-culture was set up between tumour cells (breast/prostate) and osteoblasts. The co-culture did not show significant influence on breast cancer cells and osteoblasts, but showed to

trigger the intake of Endo180 from the medium by prostate cancer cells. More interpretations of these results can be found in Chapter 7.

The final chapter summarises the entire project: the development of Endo180 ELISA and its feasibility in clinical application to measure Endo180 levels in patient plasma and conditioned media, further validation of Endo180 as a biomarker for advanced breast cancer, and the investigation into the mechanism of the release of Endo180. It also highlights the wider implementation of this project to the cancer research field, and points out the future research directions which could deepen the understanding of the function of Endo180 in the context of tumour metastasis.

2 Literature review

2.1 Breast cancer

2.1.1 Overview

Breast cancer is the most common carcinoma and the major cause of tumour-induced deaths among women (Boyle and Levin, 2008). Worldwide it is estimated that about 1.7 million women were diagnosed with breast cancer in 2012, accounting for 11.9% of all diagnosed cancer cases (Ferlay et al., 2013). In the United Kingdom (UK), breast cancer is the most common cancer type, accounting for 15% of all new cancer cases, the incidence rates have increased by 3% over the last decade and the lifetime risk of being diagnosed with the disease is 1 in 8 for women and 1 in 870 for men (Cancer Research UK, 2015). In the United States, an estimated 252,710 new cases of breast cancer will be diagnosed among women in 2017, while approximately 40,610 women are expected to die from breast cancer (American Cancer Society, 2017).

Breast cancer usually originates from the ductal epithelium of the mammary gland (~68.4% of total cases), while a minority (~9%) originate from the lobular epithelium (Sariego, 2010). Due to differences in breast cancer type, stage, treatment, the patient's geographical location and economic/social status, the prognoses for breast cancer are subject to high levels of variability. The five-year cumulative relative survival rate in England is 87.2% for the 50 – 59 age group, and a lower survival rate of 82.9% for the younger age group (0 – 49) (Møller et al., 2010). Although more than 90% of women diagnosed with breast cancer at its earliest stage can survive their disease for at least five years, this figure is dramatically reduced to approximately 15% for those women diagnosed with the advanced stages of the disease. In the advanced cases of breast cancer, the tumour will have typically spread to distant sites beyond the axillary lymph nodes (Cancer Research UK, 2014a). This spread involves the dissemination of tumour

cells to soft tissues in visceral organs, like the liver and lungs, and the much harder mineralised osteoid that makes up skeletal bone (see Section 2.2 for a detailed description of metastasis).

2.1.2 Classification and treatment of breast cancer

In order to select the best treatment, breast cancer is divided into categories according to the following factors: the histopathological type, the grade of the tumour (the microscopic similarity of breast cancer cells to normal breast tissue), the stage of the tumour (TNM system), receptor status, and the expression of proteins and genes (Edge and Compton, 2010, Jönsson et al., 2010, Kosir, 2016). Depending on several factors such as the stage of disease and the hormonal receptor status of the patient, patients was given one type or a combination of different types of treatments, which includes surgery, chemotherapy, radiotherapy, adjuvant hormonal therapy and immune therapy (Petit et al., 2011).

The number staging of breast cancer, based on the TNM classification system, is widely used in clinical practice. It divides breast cancer into five stages according to the size of tumour (T), whether cancer cells have spread to the nearby lymph nodes (N), and whether the tumour has metastasised (M). Breast cancer patients with larger tumour size, nodal spread, and metastasis usually have a larger stage number and a worse prognosis (Cancer Research UK, 2014b).

Stage 0 is a pre-cancerous or marker condition, either lobular carcinoma *in situ* (LCIS) or ductal carcinoma *in situ* (DCIS). Since lobular carcinoma *in situ* is not a true cancer or pre-cancer, often no immediate or active treatment is recommended. But because having LCIS increases the risk of developing invasive cancer, close monitoring is very important. This usually includes a yearly mammogram and a clinical breast exam (National Comprehensive Cancer Network, 2016a). In most cases, a woman with DCIS

can choose between breast-conserving surgery (BCS) followed by radiation therapy and simple mastectomy (Wolff et al., 2014, Morrow et al., 2015, National Comprehensive Cancer Network, 2016b).

Breast tumours at stage 1 – 3 are within the breast or regional lymph nodes. At stage 1, the tumour is relatively small (2 cm or smaller), and breast cancer cells have not spread to the lymph nodes or only spread to a tiny area of the sentinel lymph node. At stage 2, the tumour is large (2 – 5 cm) and/or has spread to a few (one to three) nearby lymph nodes. At stage 3, the tumour is larger (> 5 cm) or growing into nearby tissues (the skin over the breast or the muscle underneath), or the cancer has spread to many (more than three) nearby lymph nodes. Stage 1 – 2 breast cancer patients can be treated with either BCS or mastectomy followed by radiation therapy. The lymph nodes will be checked, either with a sentinel lymph node biopsy or an axillary lymph node dissection. Stage 3 breast cancer patients are treated with chemotherapy before surgery (BCS or mastectomy depending on whether the tumour shrinks enough after chemotherapy, and an axillary lymph node dissection), and radiation therapy is given after surgery. Adjuvant hormone therapy is recommended to all patients who have a hormone receptor-positive (estrogen or progesterone) invasive breast cancer. Chemotherapy is recommended for all patients with hormone receptor-negative invasive breast cancers, and for patients with hormone receptor-positive tumours who might additionally benefit from having chemotherapy along with their hormone therapy, based on characteristics of the tumour. Human epidermal growth factor receptor-2 (HER2) positive breast cancer patients are usually given trastuzumab, a humanised anti-HER2 monoclonal antibody (mAb), along with chemotherapy as part of their treatment (Wolff et al., 2014, Morrow et al., 2015, National Comprehensive Cancer Network, 2016b).

At stage 4, breast cancer cells have spread beyond the breast and lymph nodes to other parts of the body such as the bones, lungs, liver or brain, referred to as metastatic breast cancer or metastasis (see Section 2.2). Systemic therapy is the main treatment for stage 4 breast cancer. This includes chemotherapy, hormone therapy, targeted therapies (drugs that identify and attack cancer cells more precisely, while doing little damage to normal cells), immunotherapy (cytokines, humanised mAbs, or tumour vaccines that boost the immune system), radiopharmaceuticals, or some combination of these treatments, which can shrink tumours, improve symptoms, and help patients live longer, but cannot cure the cancer (Wolff et al., 2014, Morrow et al., 2015, National Comprehensive Cancer Network, 2016b).

Breast cancer patients with bone metastases are usually treated with bisphosphonates and/or the humanised mAb denosumab, to relieve the skeletal-related events (SRE) and pain caused by metastatic cancer cells (Wolff et al., 2014, Morrow et al., 2015, National Comprehensive Cancer Network, 2016b). 1) Bisphosphonates are a group of drugs that can slow down the action of osteoclasts, which are often overactive when cancer spreads to the bones (Fleisch, 2001). Bisphosphonates can help relieve symptoms by slowing down bone damage and reducing hypercalcemia caused by metastatic cancer (Cremers and Papapoulos, 2011)(please refer to Section 2.5 for a detailed overview of bisphosphonates). 2) Denosumab is a mAb that can inhibit receptor activator of NF-κB ligand (RANKL), which binds to receptor activator of NF-κB (RANK) to activate bone resorption. This treatment prevents the bone degrading osteoclasts from being turned on. It has been approved by FDA, and is recommended by NICE, for the treatment of bone metastases from breast cancer (National Institute for Health and Care Excellence, 2012, National Cancer Institute, 2013). Studies have shown that denosumab can help prevent or delay SRE in advanced breast cancer patients with bone metastases, such as

pathological fractures, with a similar efficacy as zoledronic acid (Stopeck et al., 2010). Denosumab can be used as a replacement when zoledronic acid is no longer working (American Cancer Society, 2016).

2.1.3 Cell-line models for breast cancer research

Breast cancer cell lines in continuous culture are the basic need for breast cancer research. Scientists made the attempt to culture breast tumour cells as far back as the 1930s (Cameron and Chambers, 1937). It was not until the 1950s that the first human breast carcinoma cell line in successful long-term culture, BT-20, was reported (Lasfargues and Ozzello, 1958). More than forty cell lines were established within the next 20 years, most of which are currently maintained either in the frozen state or by serial passages (Engel and Young, 1978). Sixty-five breast cancer cell lines with detailed descriptions have been reported by (Lacroix and Leclercq, 2004).

The MDA-MB-231 cell line was established from a single sample of pleural effusion obtained in 17 October 1973, from a 51-year-old white woman who had had a right radical mastectomy in January 1969 for a poorly differentiated tumour tending toward papillary configuration and tubule formation (Cailleau et al., 1974). MDA-MB-231 cells express moderate levels of Endo180 (see Section 2.4 for detailed overview of Endo180) and have been used in previous studies as a model of invasive breast cancer (Sturge et al., 2003, Wienke et al., 2007).

The MCF-7 cell line is a human breast adenocarcinoma cell line established from the pleural effusion from a 69 year old female Caucasian suffering from a breast adenocarcinoma (Soule et al., 1973). The MCF-7 cell line retains several characteristics of differentiated mammary epithelium including ability to process estradiol via cytoplasmic ER and the capability of forming domes, and the cells express the Wingless-type MMTV integration site family 7B (WNT7B) oncogene (Huguet et al.,

1994). MCF-7 cells express wild type and variant ER as well as progesterone receptor (PR) (Zhang et al., 1993). Under standard culture conditions, MCF-7 cells express low to negligible levels of Endo180 (Sturge et al., 2003, Wienke et al., 2007). Therefore, researchers have used MCF-7 cells that were transfected to overexpress wild-type or mutant Endo180 protein to investigate the role of Endo180 in tumour cell migration and extracellular matrix (ECM) remodelling (Wienke et al., 2003, Sturge et al., 2006).

The MCF10A cell line is a spontaneously immortalised human breast epithelial cell line derived from mastectomy tissue from a 36-year-old parous premenopausal woman with fibrocystic disease and no family history of breast malignancy (Soule et al., 1990). The MCF10A cell line is a non-tumorigenic epithelial cell line that is positive for epithelial sialic acid mucins, cytokeratin and milk-fat globule antigen (Soule and McGrath, 1991). They exhibit three-dimensional (3D) growth in collagen, and will form domes when grown to confluence and show no signs of terminal differentiation or senescence following long term culture (Tait et al., 1990). MCF10A cells display biological responses to treatment with insulin, glucocorticoids, cholera enterotoxin and epidermal growth factor (EGF). By electron microscopy MCF10A cells can be seen to have the characteristics of luminal ductal cells but not of myoepithelial cells. They also express breast specific antigens as detected by positive reaction with MFA-breast and MC5 mAbs. The calcium content of the medium exerts a strong effect on the morphology of the cells (Soule and McGrath, 1991). Immunoassay has revealed that MCF10A cells express negligible levels of Endo180 (Wienke et al., 2007).

2.2 Breast cancer metastasis

The final stage of most malignancies is metastatic dissemination, in which the tumour cells spread from the original organ to other adjacent or more distant tissue sites (Klein, 2008, Steeg, 2016). The most common destinations for breast cancer metastasis are the

lungs, liver, brain, and bones, with distinct patient-specific patterns of organ dissemination observed (Weigelt et al., 2005).

Metastatic breast cancer can be treated, sometimes for many years, but it can hardly be cured. Distant metastases are the cause of about 90% of deaths due to breast cancer (Bendre et al., 2003). It has been shown that the five-year survival rate is 22% in patients diagnosed with stage 4 (metastatic) breast cancer, which is considerably lower than that of the earlier stages: at stage 3, the five-year relative survival rate is 72%, and at stage 2 is over 90% (American Cancer Society, 2017).

As is shown in Figure 2.1, after carcinoma cells form a tumour mass in the primary tissue, they need to complete a multiplex pathway before successfully migrating and invading into the secondary tissue site, a process commonly referred to as the invasion-metastasis cascade (Valastyan and Weinberg, 2011). It has been suggested that breast cancer cells could acquire distinct traits to complete the invasion-metastasis cascade by activating the epithelial-mesenchymal transition program (Yang and Weinberg, 2008).

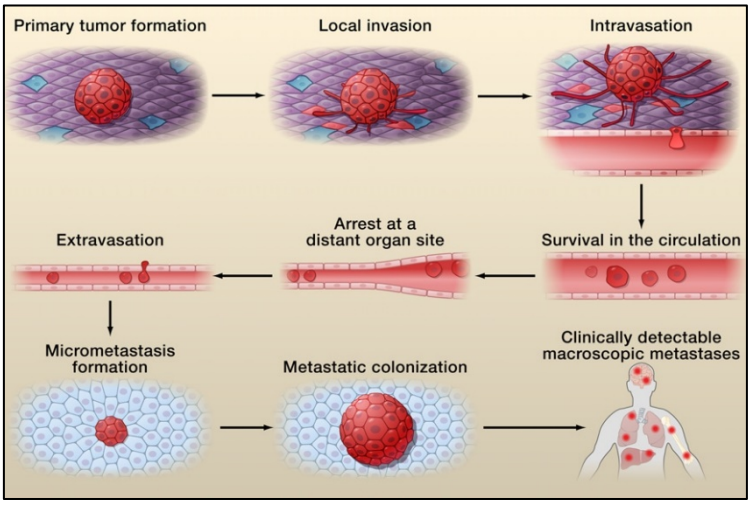


Figure 2.1 The invasion-metastasis cascade

Metastases occur after a series of cell-biological events, which are collectively termed as invasion-metastasis cascade, during which tumour cells exit their primary sites of growth (local invasion, intravasation), translocate systemically (survival in the circulation, arrest at a distant organ site, extravasation), and adapt to survive and thrive in the foreign microenvironments of distant tissues (micrometastasis formation, metastatic colonisation). Carcinoma cells are depicted in red. Reproduced from Valastyan & Weinberg (2011) with permission from Elsevier.

2.2.1 Epithelial – Mesenchymal Transition (EMT)

2.2.1.1 Overview

The term "*epithelial to mesenchymal transition*", and its reverse procedure, "*mesenchymal to epithelial transition*"(MET), was first used in cancer research area in the 1990s (Bae et al., 1993). Before this, EMT was referred to as "epithelial-to-mesenchymal transformation" or "epithelium-mesenchyme inter-conversion", which were terminologies used by developmental biologists (Thiery, 1984).

EMT is a biological process during which an epithelial-originated cell loses its epithelial phenotype and assumes a mesenchymal phenotype, such as the capability of migration and invasion, resistance to apoptosis, and expression of ECM components (Kalluri and Weinberg, 2009). EMT is divided into three types according to the different biological functions they involved: Type 1 EMT occurs during embryonic development, and is involved in critical processes such as gastrulation; Type 2 EMT participates in wound healing, tissue regeneration and organ fibrosis in mature tissues; Type 3 EMT takes place in epithelial cancer cells and is associated with cancer progression and metastasis. Type 1 and 2 EMTs are physiological EMTs, which are important in organism development and epithelia maintenance. Type 3 EMT is a pathological EMT, which mediates the morphology and phenotype changes of cancer cells, to facilitate their migration and invasion (Foroni et al., 2012).

Both physiological and pathological EMTs have similar molecular mechanisms. Cells that have undergone EMT usually show decreased E-cadherin expression and increased vimentin and N-cadherin expression, which have been used as markers for the detection of EMT (Perl et al., 1998, Beavon, 2000). EMT can be induced by mesenchymal cell-released ECM components or growth factors, such as transforming growth factor–beta (TGF-β), hepatocyte growth factors (HGF), fibroblast growth factors (FGF), epithelial

growth factors (EGF) and insulin-like growth factors (IGF) (Massagué, 2008, Yang and Weinberg, 2008, Roussos et al., 2010). Signal transduction pathways involved in EMT include WNT, Notch, nuclear factor-kappaB (NF-κB) and integrin signalling pathway, as well as E-cadherin repressing signalling pathways mediated by a number of transcription factors (Wang et al., 2006, Bailey et al., 2007, Moreno-Bueno et al., 2008, Yang and Weinberg, 2008, Canesin et al., 2015). A detailed list of EMT related markers can be found in Table 2.1.

Table 2.1 EMT related markers

Upregulation of	*Downregulation of*	*Activation of*	*Cell functional acquisition*
N-cadherin	E-cadherin	β-catenin	Increased migration
Vimentin	Desmoplakin	Smad-2/3	Increased invasion
Fibronectin	Cytokeratin	NF-κB	Increased scattering
Snail1 (Snail)	Occludin	Snail1 (Snail)	Stem-cell phenotype
Snail2 (Slug)	Claudin	Snail2 (Slug)	Drug resistance
Twist	miRNA200 family	Twist	
GSC		Zeb1, Zeb2	
FOXC2			
Zeb1, Zeb2			
MMP-2, MMP-3, MMP-9			
Integrin $\alpha_V\beta_6$			
LOX-L2, E47			

Note: Table adapted from Foroni et al. (2012) with permission from Elsevier

Matrix metalloproteinases (MMPs) also showed correlations with EMT. EMT-inducing signals such as Snail and Zeb can induce the expression of a number of MMPs, including MMP1, MMP-2, MMP-7, MMP-9 and MT1-MMP, which participate in ECM remodelling (Yokoyama et al., 2003, Miyoshi et al., 2004, Jorda et al., 2005). In turn, some MMPs such as MMP-3 and MMP-13, could induce EMT through the activation of EMT-inducing factors (Radisky et al., 2005, Billottet et al., 2008).

Many of the EMT-inducing factors and signalling pathways mentioned above were observed and identified in carcinoma cells. For example, E-cadherin repressors Snail 1 and Snail 2 are shown to be correlated with tumour recurrence in breast, colorectal, and

ovarian cancer (Moody et al., 2005). This suggests that EMT has a strong correlation with cancer progression (Roussos et al., 2010).

2.2.1.2 EMT and cancer progression

One of the most significant effects of EMT in cancer is the promotion of metastasis. To invade into the stromal layer, tumour cells have to break through the basal lamina (also termed basement membrane, BM) (Felipe Lima et al., 2016). The BM is a special type of ECM that plays an important role in separating epithelial and stromal tissue compartments (Valastyan and Weinberg, 2011). This type of epithelial cell sheets has strong E-cadherin mediated intercellular junctions that can prevent individual cells from escaping from epithelial tissue, as well as block the carcinoma cells from invading into the stroma (Beavon, 2000). EMT overcomes this blockage in two aspects: the first one is allowing epithelial cells to lose their basal-lateral-apical polarity and gain front-rear polarity, which is a typical characteristic of mesenchymal cells; the second one is breaking junctions between epithelial cells. This transaction gives individual epithelial cells multiple mesenchymal properties, including the potential of increased invasiveness (Thiery et al., 2009).

In addition, EMT also participates in many other processes associated with tumour development and progression. EMT facilitates tumour cells to gain the capacity of resistance to senescence and apoptosis (Felipe Lima et al., 2016). Evidence for this comes from the fact that the non-tumorigenic mouse mammary epithelial cell line NMuMG underwent EMT and showed resistance to apoptosis after chronic TGF-β treatment (Gal et al., 2008). The program of EMT induced by TGF-β also requires the assistant of E-cadherin repressors such as Snail, which confers resistance to apoptosis (Barrallo-Gimeno and Nieto, 2005).

EMT enhances the resistance of tumour cells to chemotherapy and immunotherapy (Thiery et al., 2009). For example, colon carcinoma epithelial cell lines with a mesenchymal morphology that express EMT markers are resistant to oxaliplatin treatment (Yang et al., 2006). Ovarian carcinoma epithelial cell lines that lose their epithelioid phenotype and express EMT markers are resistant to paclitaxel (Kajiyama et al., 2007). Similarly, breast cancer cell line MDA-MB-434, which has an EMT-like and high invasive phenotype, is also resistant to paclitaxel (Cheng et al., 2007). In the same breast cancer cell line, EMT marker twist and its target genes are up-regulated, and the suppression of Twist can partially reverse the resistance (Li et al., 2009). Correspondingly, overexpression of negative regulators of EMT, such as miRNA-200c, can increase chemotherapeutic sensitivity (Cochrane et al., 2009). EMT also contributes to immunotherapy resistance of dendritic cells, and can induce escape from immune surveillance, immunosuppression, inflammation, and confers stem cell properties on tumour cells (Mani et al., 2008, Kudo-Saito et al., 2009). These studies demonstrate that EMT is an important feature within the tumour microenvironment, which could be responsible for drug resistance of tumour cells.

It has been found that hypoxia is one of the physiological drivers that are closely correlated with EMT in cancer (Lamouille et al., 2014). Hypoxia can induce EMT through a number of distinct mechanisms, including upregulation of hypoxia-inducible factor (HIF), HGF, Snail 1, Twist, and the activation of Notch, NF-κB signalling pathways (Polyak and Weinberg, 2009). The increased expression of HIFs in primary tumour biopsies from breast cancer patients are associated with increased risk of metastasis (Gilkes and Semenza, 2013). It has been shown *in vitro* that hypoxia can induce EMT and repress ER gene expression in the breast cancer cell line MCF-7 (Cannito et al., 2008, Yao-Borengasser et al., 2015). The inhibition of ER could also

promote EMT and cause the resistance of breast cancer cells to hormonal therapy (Bouris et al., 2015). Hypoxia also induces the expression of collagen-modifying enzymes, such as lysyl oxidase (LOX), which causes stiffening of ECM and is essential for tumour metastasis (Erler et al., 2006, Postovit et al., 2008, Levental et al., 2009, Cox et al., 2013). Matrix stiffness is also considered to be a driver of EMT and tumour metastasis (Wei et al., 2015). This suggests a correlation of hypoxia, ECM stiffness and EMT in tumour progression.

Studies have also revealed the correlations between EMT and breast cancer progression. It has been shown that the increased expression of EMT-related E-cadherin repressors, such as Snail, correlates with the decrease in relapse-free survival in women with breast cancer (Moody et al., 2005). The expression profiles of the EMT inducers have also been associated with the histological grade and a basal-like phenotype in breast cancer (Blanco et al., 2002, Sarrio et al., 2008). TGF-β is shown to induce EMT and bone metastasis of breast tumour cells via the TGF-β / Smad signalling pathway (Deckers et al., 2006). Ionising radiation, which mimics the pre-neoplastic progression of breast carcinoma, can induce TGF-β mediated EMT in non-malignant mammary epithelial cells, and this process is facilitated by β_3 integrin (Galliher and Schiemann, 2006, Andarawewa et al., 2007). With regard to the increased activations of proteolytic activity and invasiveness, the EMT program induced by SIM2 knockdown in MCF-7 cells was associated with increased MMP-2 levels (Laffin et al., 2008).

2.3 Tumour markers

2.3.1 Overview

Tumour markers, one type of biomarker, are substances that are associated with cancerous conditions (Nagpal et al., 2016). They are made by normal cells as well as by tumour cells, and can be found in the tumour tissue, blood, urine, stool, or other

tissues and bodily fluids of cancer patients (Bigbee and Herberman, 2013). A tumour marker may help to diagnose cancer, plan treatment, or find out how well the treatment is working or there is disease recurrence. Examples of currently used tumour markers include carcinoma antigen (CA-125) in ovarian cancer, carcinoembryonic antigen (CEA) in colon cancer, and prostate-specific antigen (PSA) in prostate cancer (Andriole et al., 2009, Schroder et al., 2009, Buys et al., 2011, Cramer et al., 2011). Biomarkers for breast cancer are important in predicting the prognosis of breast cancer patients, and are useful in deciding the necessity and type of therapy for each individual personalised medicine as discussed in the following section.

2.3.2 Tumour markers for breast cancer

There are two types of tumour markers that are currently used or potentially useful in breast cancer clinical practice, including tissue-based markers and serum-based markers.

The most widely-used tissue-based markers for breast cancer are ER, PR and HER2 (Bardou et al., 2003, Ross et al., 2003, Duffy, 2006a). Measurement of ER and PR levels is essential in evaluating the response of early and advanced breast cancer patients to hormone therapy, and is helpful in assessing the short-term prognosis in patients with newly diagnosed breast cancer (Ravdin et al., 1992, Early Breast Cancer Trialists' Collaborative Group, 1998, Mirza et al., 2002, Early Breast Cancer Trialists' Collaborative Group, 2005). HER2 level is measured to determine the necessity of treatment with trastuzumab and anthracycline-based adjuvant chemotherapy (Slamon et al., 2001, Yamauchi et al., 2001, Piccart-Gebhart et al., 2005, Romond et al., 2005). However, the hormonal biomarkers cannot always reflect the patient's response to specific therapy, due to the heterogeneity of breast tumours and their different intrinsic

features and extrinsic features, which could lead to different clinical characteristics and responses to treatment (Bertos and Park, 2011).

In comparison to tissue markers, serum markers have the advantage of non-invasive and cost-efficient measurement, which makes serial determinations easier to perform and without the need for painful biopsy procedures. CA 15-3 is a widely-used serum-based marker for breast cancer (Duffy et al., 2010). CA 15-3 is an epitope of the MUC-1 protein (also known as polymorphic epithelial mucin), which plays an important role in cell-to-ECM and cell-to-cell interactions (Duffy et al., 2004, Duffy et al., 2010). The extracellular region of the MUC-1 protein is shown to be over-expressed and less-glycosylated in malignant breast tumour cells, which can be shed into the blood and is elevated in the plasma of approximately 75% of women with metastasised breast cancer (Burchell et al., 2001, Kufe, 2009, Ferlay et al., 2013). Altered expression and glycosylation of the MUC-1 protein could contribute to tumour formation and metastasis, such as trigger tumour proliferation, enhance cell motility, and block immune cells (Schroeder et al., 2003, Carraway et al., 2005, Hattrup and Gendler, 2008). CA 15-3 and associated CA 27.29 (a different epitope on the same protein antigen product of MUC1 gene, which has enhanced sensitivity and specificity) are used as tumour markers to evaluate the response to chemotherapy and to monitor for recurrence of disease in patients with advanced breast cancer, as well as postoperative surveillance in patients with no evidence of disease (Cheung et al., 2000, Duffy, 2006b, Duffy et al., 2010, Fahmueller et al., 2013). However, both CA 15-3 and CA 27.29 levels can also be raised due to the presence of other conditions. For example, they can be elevated in patients with benign breast disease, benign ovarian cysts and hepatitis. For this reason, CA 15-3 cannot replace routine clinical assessment and diagnostic imaging in screening, and is recommended to be used in combination with conventional clinical examination

in monitoring therapy (Harris et al., 2007, Cardoso et al., 2012). Physicians should always use the CA15-3 test results in conjunction with other diagnostic test results and full medical history to make decisions about the management of their patients (Duffy et al., 2010). Routine preoperative measurement of CA 15-3 for determining prognosis in patients with early breast cancer is not recommended by National Academy of Clinical Biochemistry (Sturgeon et al., 2008).

2.3.3 Tumour markers for bone metastasis

Several biomarkers for bone turn over are also used as tumour markers for metastatic bone disease in breast cancer and other cancer types (Ferreira et al., 2015). These include carboxy-terminal cross-linking telopeptide of type I collagen (CTX) and bone alkaline phosphatase (BALP) (Ramaswamy et al., 2000, Andreas Compare Cloos et al., 2003, Leeming et al., 2006, Lipton et al., 2011).

CTX is an enzymatic hydrolysis product of type 1 collagen that contains cross-linking regions (Garnero and Delmas, 1998). It is released when mature collagen fibrils are degraded, and is used as a specific biomarker to measure the level of bone resorption (Christgau et al., 2000). CTX shows significant responses to antiresorptive therapies, such as bisphosphonate treatment, so it is also used as a biomarker to monitor the efficacy of antiresorptive therapy, as well as to evaluate the risk of osteonecrosis for patients with bone disorders such as osteopenia and osteoporosis who are administered bisphosphonates as part of their treatment regimen (Rosen et al., 2000, Marx et al., 2007).

BALP is a tetrameric glycoprotein which is located on the plasma membrane of osteoblasts (Garnero and Delmas, 1993). BALP could have the membrane-anchoring site cleaved by phospholipase and enter the circulation as dimers or membrane vesicles (Howard et al., 1987, Fedde, 1992). Serum BALP reflects the osteogenic activity, and

was shown to be more sensitive and reliable than total alkaline phosphatase as a biomarker of bone metabolism (Stepan et al., 1978). An immunoradiometric assay which measures serum BALP levels has been developed and implemented in the diagnosis and assessment of metabolic bone disease, as well as to monitor the clinical response to antiresorptive treatments like bisphosphonates (Garnero and Delmas, 1993).

However, both CTX and BALP are not specific for metastatic bone disease and there are several other factors that could cause increased levels of these biomarkers (Rosen et al., 2000, Seibel, 2005). Therefore, it is necessary to develop a more sensitive breast cancer biomarker that can accurately predict the stage of the disease and be measured easily and quickly to help indicate whether the disease is progressing to improve the diagnostic limitations of currently used biomarkers.

2.3.4 Techniques used in measurement of serum-based tumour makers

Immunoassay (also knowns as immunodiagnostic or immunoscreening in clinical practice) is the most commonly used method in determining tumour markers in patients' body fluids such as serum and urine (Sharma, 2009). In terms of the quantitative measurement of a tumour marker, ELISA is a useful tool and is widely used in determining serum-based tumour markers in clinical practice (Powers and Palecek, 2012).

ELISA was developed from radioimmunoassay and was first described and published by two scientific research groups independently and simultaneously (Yalow and Berson, 1960, Engvall and Perlmann, 1971, van Weemen and Schuurs, 1971). The successful development of this immunoassay is also inseparable from the contribution of scientists from a number of related fields. Technique enabling the chemical link between detecting antibodies and signal developing enzymes, the activities of which produce a measurable signal with appropriate substrates, was developed by a group in Villejuif

and then optimised by Avrameas (1969) and Nakane & Pierce (1967). The development of the method to produce antigen-specific mAbs led to the use of this technique to produce probes for detecting individual molecules in complex protein mixtures or tissue samples (Kohler and Milstein, 1975). The traditional ELISA technique developed into several different kinds of modified assay types, including enzyme-based assays that use fluorescence, electrochemiluminescent (ECL), quantitative PCR reporters or gold nanoparticles (Schweitzer et al., 2000, de la Rica and Stevens, 2012) to create quantifiable signals, and non-enzyme assays that linked to some non-enzymatic reporter. These technological developments can have distinct advantages compared to classic ELISA: including higher sensitivities for the detection of lower abundance markers, and the measurement of multi-component biomarker signatures or conformational changes in a biomarker by multiplexing (Leng et al., 2008).

Various types of ELISA have been developed (Figure 2.2) that share similar basic principles and procedures, which can be summarised as follows: 1) antigens are directly or indirectly immobilised to the surface of polystyrene microplate wells; 2) irrelevant protein is added to block all uncovered surface of the microplate wells; 3) incubation with antigen-specific antibodies (labelled with a signal-generating enzyme or biotin) that affinity-bind to the antigens; 4) detection of the signal generated by enzyme with the added substrate (the signal observed is proportional to the amount of antigen in the sample). Washing between these steps is essential to ensure that the performance of the assay is optimised by removing the majority of non-specific binding that would otherwise cause high levels of background noise.

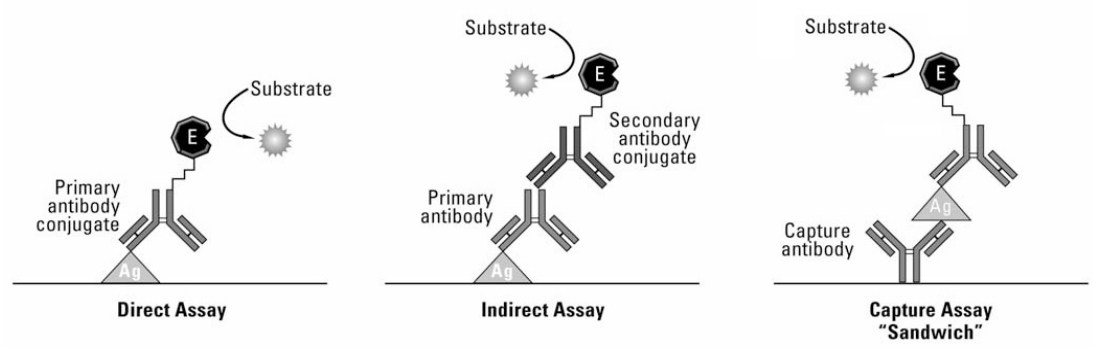


Figure 2.2 Typical classificaitons of ELISA

1) Direct assay: antigens are directly attached to the plate by passive adsorption, the detection antibody is labelled of with an enzyme which can generate detectable signals when substrate is added; 2) Indirect assay: antigens are directly attached to the plate, an additional probing step using secondary antibody that is labelled with a detectable tag is involved in the detection procedure; 3) Capture (Sandwich) assay: antigen-specific antibody is adsorbed onto the plastic, which in turn binds and immobilizes the antigen upon incubation with the sample, the detection antibody is labelled of with an enzyme which can generate detectable signals when substrate is added. Reproduced from *ELISA technical guide and protocols* (Thermo Fisher Scientific, 2010)

Previous studies have shown that the value of Endo180 as a cancer biomarker is evident from its elevated expression in tumour tissue including breast, prostate, glioblastoma multiforme, head and neck and liver being linked to clinical grade, disease progression and/or decreased survival (Sulek et al., 2007, Wienke et al., 2007, Kogianni et al., 2009, Huijbers et al., 2010, Gai et al., 2014, Rodriguez-Teja et al., 2015a, Rodriguez-Teja et al., 2015b). Moreover, a recent study has shown that the Endo180 receptor ectodomain is released from breast tumour cells and that measurement of this soluble form of Endo180 in plasma, alone or in combination with CA 15-3 antigen, can provide a more accurate method for the classification of metastatic disease (Palmieri et al., 2013a). In that study, the release of soluble Endo180 levels into serum and conditioned medium of breast cancer cell cultures was measured using semi-quantitative immunoblot analysis. It was also observed in the same study that bisphosphonate treatment is correlated with a reduction in the levels of Endo180 in the serum of advanced breast cancer patients with metastatic bone disease. To study the molecular mechanism(s) and clinical applications of Endo180 release further in the context of breast cancer metastasis, it would be a significant advantage if a quantitative ELISA is available.

2.4 Endo180

2.4.1 Overview

Endo180 (endocytic receptor 180), also known as MRC2 (mannose receptor C-type 2), CD280 (cluster of differentiation 280), uPARAP (urokinase-type plasminogen activator receptor associated protein), CLEC13E, KIAA0709 and TEM9, was first identified in the early 1990s as a type I transmembrane collagen receptor that can be constitutively recycled (Isacke et al., 1990). In later studies, Endo180 was confirmed as a member of the mannose receptor family, which contains mannose receptor C-type 1 (MRC 1, CD206), the M-type phospholipase A_2 receptor (PLA_2R) and DEC-205 (Wu et al., 1996). Endo180 has also been designated as uPARAP, due to its function as a component of the tri-molecular complex which contains pro-urokinase plasminogen activator (uPA) and the urokinase plasminogen activator receptor (uPAR) (Behrendt et al., 2000). Since its identification, a number of reviews have been published, which provided comprehensive introductions of relative concepts and research progress about the biological mechanisms and clinical functions of Endo180 (Engelholm et al., 2001a, East and Isacke, 2002, Behrendt, 2004, Leitinger and Hohenester, 2007, Llorca, 2008, Engelholm et al., 2009, Melander et al., 2015, Sturge, 2016).

2.4.2 Structure of Endo180

Endo180 has similar structure type as other members of mannose receptor family, which can be subdivided into the extracellular domain, the transmembrane domain, and the intracellular domain (Figure 2.3). The extracellular domain of Endo180 is 150 kDa in atomic mass, and consists of several different structural elements, which includes a cysteine-rich domain (CRD), a fibronectin type II-like (FNII) domain and eight C-type lectin domains (CTLD 1 – 8). Glycosylation appears at certain sites of the extracellular domain. The transmembrane domain is a single transmembrane domain like other

proteins in mannose receptor family. Endo180 also has an intracellular domain which has several predicted phosphorylation sites within its 42 amino acid residues (Behrendt et al., 2000, Sheikh et al., 2000, East et al., 2002, Wienke et al., 2003).

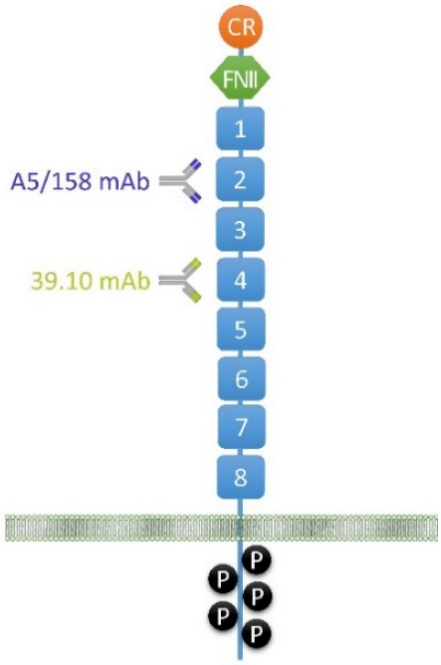


Figure 2.3 Structure of Endo180
The ectodomain (150 kDa) of Endo180 is composed of a cysteine-rich domain (CRD), a fibronectin type II-like domain (FNII) and eight C-type lectin domains (CTLD 1-8). Endo180 also has a single transmembrane domain and a cytoplasmic tail which has several predicted phosphorylation sites within its 42 amino acid residues. Several antibodies that can bind to human Endo180 have been raised: mouse anti-human mAb A5/158 binds to CTLD-2, mouse anti-human mAb 39.10 binds to CTLD-4.

A number of antibodies that can bind to different epitopes of human Endo180 have been generated: mouse anti-human mAb E1/183 that binds to the CRD (Isacke et al., 1990), mouse anti-human mAb A5/158 that binds to the CTLD-2 (Sheikh et al., 2000, Sturge et al., 2003), mouse anti-human mAb 39.10 that binds to the CTLD-4 (Wienke et al., 2007, Palmieri et al., 2013a), and rabbit polyclonal antibody (pAb) CAT-2 that binds to the intracellular domain (Sturge et al., 2007). E1/183 and A5/158 can only bind with non-denatured Endo180 in which the disulphide bonds have not been reduced, while 39.10 and CAT-2 can detect Endo180 under both reducing and non-reducing conditions.

2.4.3 Coding and expression of Endo180

The coding gene of *Endo180* (GenBank accession number: *AF134838.1*) is located on chromosome *17q24.2*, it has 36 coding exons and is 67.39 kb in size. It also contains the similar conservative intron-exon boundaries as to the coding genes of other members in the mannose receptor family, such as CD206 and PLA_2R (Wu et al., 1996, Behrendt et al., 2000, Sheikh et al., 2000, Wienke et al., 2003).

Endo180 is expressed in a wide range of cell lines, with high expression levels in cells of a mesenchymal origin, such as fibroblasts and osteoblasts. Table 2.2 shows Endo180 expression status of different cell lines that have been previously reported.

2.4.4 Regulation of Endo180 expression

Because of the role of Endo180 in ECM remodelling, it was presumed that Endo180 is correlated with tumour invasion. As a result, many factors that stimulate EMT-like responses in tumour progression have been investigated as potential regulators of Endo180 expression in tumour cell lines (Thiery, 2002). In breast cancer cell line MCF-7, several factors including FGF, HGF, IGF-II, PDGF and TGF-β_1 were investigated for their ability to induce the expression of Endo180 (Wienke et al., 2007). Among these, TGF-β_1 was the only factor that significantly upregulated Endo180 expression. Moreover, the cells with an upregulation of Endo180 expression induced by TGF-β_1 treatment scattered away as single cells from the MCF-7 epithelial sheets and displayed a mesenchymal morphology with Endo180 strongly localised to sites of cell-ECM contacts (Figure 2.4). TGF-β_1 was also shown to upregulate the expression of Endo180 in human glioma cell line U87MG, and this effect was inhibited by the suppression of Smad3 (Huijbers et al., 2010). Furthermore, TGF-β receptor-1 kinase inhibitor VII blocked Endo180 expression in prostate tumour cell line and primary human osteoblasts co-cultures (Caley et al., 2012).

Table 2.2 Endo180 status in different cell lines

Cell Line	*Cell type*	*Species*	*Status*	*References*
F1084	embryonic, fibroblast	human	+	Isacke et al. (1990)
U937	haematopoietic, monocyte	human	+	Behrendt et al. (2000)
AG1523	mesenchymal, fibroblast	human	+	Sheikh et al. (2000)
Flow2000	mesenchymal, fibroblast	human	+	Sheikh et al. (2000)
HMEC-1	mesenchymal, fibroblast	human	+	Sheikh et al. (2000)
MRC-5	mesenchymal, fibroblast	human	+	Sheikh et al. (2000)
DMEC	vascular, endothelial	human	+	Sheikh et al. (2000)
HUVEC	vascular, endothelial	human	+	Sheikh et al. (2000)
HepG2	hepatic, normal	human	-	Sheikh et al. (2000)
Hofbauer	mesenchymal, placental	human	+	Sheikh et al. (2000)
Primary Cells	haematopoietic, macrophage	human	+	Sheikh et al. (2000)
RPM-MC	dermis, melanoma	human	+	Sheikh et al. (2000)
NIH-3T3	mesenchymal, fibroblast	mouse	+	Howard & Isacke (2002)
MG63	bone, osteosarcoma	human	+	Howard & Isacke (2002)
MEF	embryonic, fibroblast	mouse	+	East et al. (2003)
Primary Cells	dermis, fibroblast	mouse	+	Engelholm et al. (2003)
MDA-MB-231	myoepithelial, breast carcinoma	human	+	Sturge et al. (2003)
MCF-7	epithelial, breast carcinoma	human	-	Wienke et al. (2003)
MC615	cartilage, chondrocyte	mouse	+	Howard et al. (2004)
HT1080	connective, fibrosarcoma	human	+	Howard et al. (2004)
HEK293	embryonic, kidney	human	+	Fernández et al. (2005)
Gingival cells	gingival, fibroblast	human	+	Honardoust et al. (2006)
HaCat	dermal, keratinocyte	human	+	Honardoust et al. (2006)
BE	epithelial, colorectal carcinoma	human	+	Sturge et al. (2006)
Primary cells	Haematopoietic, dendritic	human	+	Butler et al. (2007)
BMM	haematopoietic	mouse	-	Sturge et al. (2007)
BMSC	fibroblast	mouse	+	Sturge et al. (2007)
HSC	hepatic, stellate	rat	+	Mousavi et al. (2009)
LX-2	hepatic, stellate	human	+	Mousavi et al. (2009)
M1-4	hepatic, stellate	mouse	+	Mousavi et al. (2009)
BSC	hepatic, stellate	rat	+	Mousavi et al. (2009)
MFBY2	hepatic, stellate	rat	+	Mousavi et al. (2009)
U87MG	brain glial, glioma	human	-	Huijbers et al. (2010)
SF188	brain glial, glioma	human	+	Huijbers et al. (2010)
Primary cells	myofibroblast	mouse	+	Shi et al. (2010)
LNCaP	epithelial, prostate carcinoma	human	-	Madsen et al. (2011)
CaCo-2	epithelial, colorectal carcinoma	human	-	Madsen et al. (2011)
HeLa	epithelial, cervical carcinoma	human	+	Madsen et al. (2011)
LLC	epithelial, lung carcinoma	mouse	-	Madsen et al. (2011)
B16F10	dermal, melanoma AG1523	mouse	+	Madsen et al. (2011)
HGF-1	gingival, fibroblast	human	+	Madsen et al. (2011)
RDNCTC2472	connective tissue, sarcoma	mouse	+	Madsen et al. (2011)
F1084	embryonic, fibroblast	human	+	Madsen et al. (2011)
Fibrocytes	mesenchymal	human	+	Bianchetti et al. (2012)
RC-165N	epithelial, prostate carcinoma	human	+	Caley et al. (2012)
RC-92a	epithelial, prostate carcinoma	human	+	Caley et al. (2012)
RC-58T	epithelial, prostate carcinoma	human	+	Caley et al. (2012)
PC-3	epithelial, prostate carcinoma	human	+	Caley et al. (2012)
DU145	epithelial, prostate carcinoma	human	+	Caley et al. (2012)
Primary cells	bone derived osteoblast	human	+	Caley et al. (2012)
RWPE-1	normal prostate epithelia	human	+	Rodriguez-Teja et al. (2015b)
BPH-1	benign prostate hyperplasia	human	+	Rodriguez-Teja et al. (2015b)
U2OS	osteosarcoma	human	+	This project

Note:

Table was adapted from Gronau (2011). Cell lines are listed together with their classification (cell type), species of origin and Endo180 expression status; Endo180 positive (+); Endo180 negative (-); corresponding original references are indicated Endo180 and cancer.

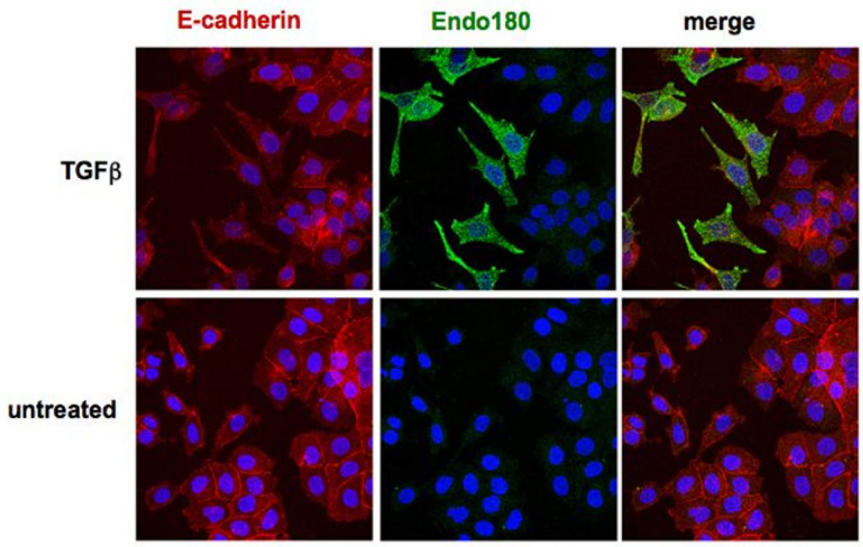


Figure 2.4 Expression of Endo180 in MCF-7 cells induced by TGF-β is associated with a migratory phenotype (unpublished data from Dr Justin Sturge)
MCF-7 cells were treated with 5 ng/ml TGF-β for 24 hours then fixed and co-immunostained with TOPRO-3 (blue), anti-E-cadherin (HECD1) (red) and anti-human Endo180 mAb (A5/158) (green). It can be seen that MCF-7 cells that express high levels of Endo180 have broken away from the epithelial sheet formed by the cells that are Endo180 negative. Endo180 accumulates in the cell uropod where it plays a role in the promotion of adhesion disassembly (Sturge et al, 2006).

TGF-β_1 has also been shown to regulate Endo180 expression in human gingival fibroblasts and HaCaT keratinocytes. Other factors shown to induce Endo180 expression in these cell types include EGF, PDGF, HB-EGF and KGF (Honardoust et al., 2006). It has also been found that interferon-γ (IFN-γ) can promote the co-expression of MT1-MMP and Endo180 in murine macrophages cultured on type-I collagen, and that interleukin-10 (IL-10) can inhibit this upregulation (Ye et al., 2010).

Endo180 expression in prostate cancer cell line PC-3 is upregulated under their direct or indirect co-culture with primary human osteoblasts (hOB) (Caley et al., 2012), or when cultured on hOB-derived or human fibroblast-derived ECM (Caley et al., 2016). In contrast, the expression of Endo180 in hOB is downregulated when they are co-cultured with PC-3 cells. This divergent regulation of Endo180 could involve a number of regulation factors released into the conditioned media by these cell types. Further works is required to determine which factors play positive/negative roles in the regulation of Endo180 expression during different biological and pathological contexts.

2.4.5 Endo180-dependent cell functions and signalling pathways

2.4.5.1 Collagen turnover – uptake and deposition

Endo180 plays an important role in collagen uptake, internalisation, and intercellular lysosomal delivery. This has been shown in experiments using Endo180-deficient fibroblasts, where both normal and Endo180-deficient fibroblasts could digest ECM, whereas Endo180-deficient fibroblasts were defective in their ability to internalise the cleaved collagen fragments when compared to their Endo180-positive counterparts (East et al., 2003, Engelholm et al., 2003, Kjøller et al., 2004, Madsen et al., 2007). The differentiation of primary hepatic stellate cells (HSC) into myofibroblast-like cells results in the increased Endo180-dependent collagen uptake (Mousavi et al., 2005, Mousavi et al., 2009). The Endo180-dependent collagen uptake and internalisation also occurs in the breast cancer cell line MCF-7 that was transfected to express wildtype Endo180 protein (Wienke et al., 2003). Moreover, when MCF-7 cells were transfected to express the mutant Endo180 that cannot be internalised, collagen uptake was inhibited (Howard and Isacke, 2002, Wienke et al., 2003). These findings further proved the key role of Endo180 recycling in collagen turnover.

Contrary to its role in the uptake of collagen fragments, the silencing of Endo180 in primary hOBs results in a marked decrease in type-I collagen deposition (Caley et al., 2012). This suggests Endo180 could work as an indirect or direct modulator of collagen production, in addition to its role as a receptor that participates in the clearance of collagen fragments from the extracellular compartment of tissues.

Endo180-dependent collagen turnover is suggested to be essential in maintaining the balance between bone formation and resorption. In support of this regulatory role of Endo180 in bone homeostasis, it has been postulated that osteoblast recruitment and the migration of osteoblast progenitors into bone resorption pits are driven by the

interactions between the Endo180 receptor on osteoprogenitors and the residual collagen fragments released during osteoclast-mediated bone degradation (Abdelgawad et al., 2014).

The role of Endo180 in collagen turnover – uptake and deposition – has also been linked to the high levels of ECM remodelling associated with tumour progression and bone metastasis (Curino et al., 2005, Wienke et al., 2007, Sturge et al., 2011, Caley et al., 2012, Engelholm et al., 2016), as discussed in more detail in Section 2.4.6.

2.4.5.2 Endo180-involved signalling pathways

Cross-linking experiments showed that Endo180 could form a complex with pro-uPA (urokinase-type plasminogen activator) and uPAR (urokinase-type plasminogen activator receptor) on the cell surface (Behrendt et al., 1993, Behrendt et al., 2000). Although the full formation procedure of the Endo180-uPA-uPAR trimolecular complex is still unknown, it is demonstrated to play an important role in directional cell migration, as silencing of Endo180 expression by siRNA or blockade of CTLD-2 or CRD in Endo180 by A5/158 or E1/183 mAbs respectively can block the directional migration of MDA-MB-231 cells (Sturge et al., 2003). As to the mechanism underlying the role of the complex, it is suggested that the association of Endo180 with uPA and uPAR could facilitate the transfer of collagen substrates to the activated collagenolytic enzymes, including MMP-1 or MMP-13, the activation of which depends on uPA-catalysed plasminogen activation (Engelholm et al., 2009).

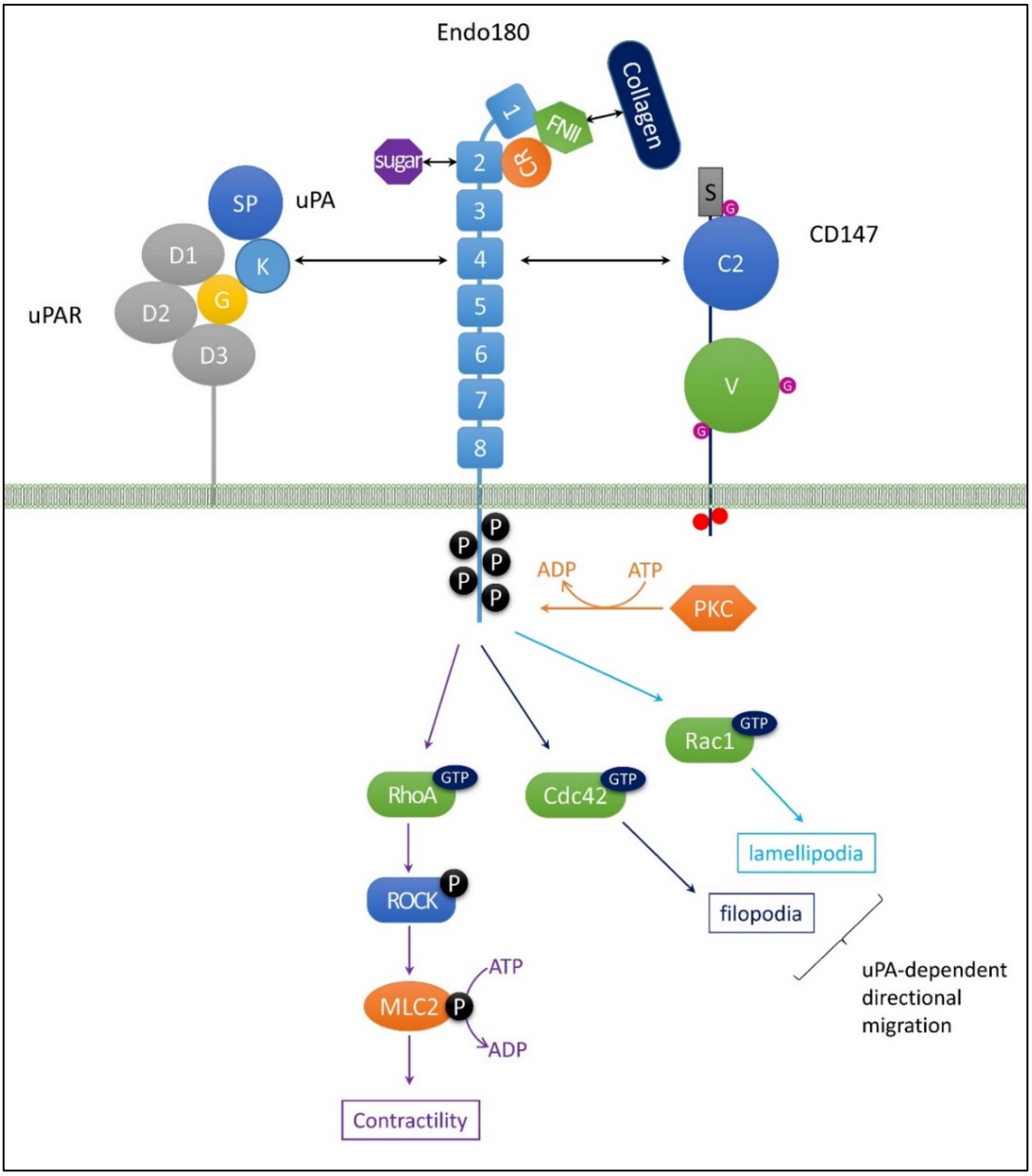


Figure 2.5 Endo180-involved signalling pathways

In Endo180, CRD, FNII and CTLD1-2 can form a loop structure, in which FN II works as a collagen binding domain and CTLD-2 as a sugar binding domain. The phosphorylation sites on the cytoplasmic tail of Endo180 are shown to be substrates of protein kinase C (PKC). Endo180 regulates the overall and spatial/temporal activation of the Rho family of small GTPases (Cdc42, Rac1, and RhoA), which plays an important role in cell migration and morphological change. The activation of Cdc42 and Rac1 is uPA dependent, and is enhanced by the trimolecular complex formed between Endo180, uPA and uPAR. Endo180 can also form a complex with CD147, which has been shown to play an important role in maintaining the epithelial phenotype of prostate epithelial cells.

Endo180: CRD – cysteine-rich domain, FNII – fibronectin type II-like domain, 1-8 – eight C-type lectin domains (CTLD 1-8), P – phosphorylation sites. **uPA**: SP – serine protease domain, K – kringle domain, G – growth factor domain. **uPAR**: D1-3 – three lymphocyte antigen-6/uPAR domains (LU domains). **CD147**: S – signal peptide, C2 – immunoglobulin (Ig)-like C2-type domain, V – Ig-like V-type domain, G – N-Glycosylation sites, red cycle – hypoxia-inducible factor (HIF) binding sites.

Endo180 is demonstrated to play an important role in the downstream signalling pathway that involves members of the Rho family of small GTPases, Cdc42, Rac1, and RhoA, which have been shown to regulate intracellular actin dynamics (Sturge et al., 2003, Sturge et al., 2006). The expression of Endo180 in breast cancer cells could enhance the uPA-mediated filopodia production and promote the rapid activation of Cdc42 and Rac1, which leads to the promotion of the chemotactic tumour cell migration (Sturge et al., 2003). Endo180 could also mediate the activation of RhoA, which activates Rho-associated protein kinase (ROCK) and leads to the phosphorylation of myosin light chain-2 (MLC2), so as to regulate cell motility (Sturge et al., 2006). Although the molecular signalling pathway involved in Endo180-dependent Rho GTPases activation has not been fully delineated, evidence from proteomic analysis carried out in Dr Justin Sturge's laboratory has indicated that Endo180 could interact with Rho guanine nucleotide exchange factors (Rho GEFs), as well as the intermediate filament component vimentin (data not shown). Studies showed that Rho GEFs and vimentin contribute to EMT in cancer progression (Liu et al., 2015, Komiya et al., 2016), and Rho GEFs are regulated by TGF-β during EMT (Osborne et al., 2014).

In a recent study, it has been proved that the CTLD-4 domain of Endo180 can interact and form a complex with CD147, also known as extracellular matrix metalloproteinase inducer (EMMPRIN), which is a member of the immunoglobulin superfamily and works as a cell adhesion modulator. Endo180-CD147 complex is shown to be a novel EMT suppressor, the disruption of which decreases cell-cell adhesion and promotes EMT in prostate epithelial cells (Rodriguez-Teja et al., 2015b).

2.4.6 The role of Endo180 in tumour progression

2.4.6.1 Endo180 in EMT suppression and promotion

The key step in the transformation of the benign tumour to metastatic tumour is the acquisition of the ability to migrate and invade by tumour cells. These tumour cells obtain mesenchymal-like properties through the EMT procedure, and are able to express large amount of proteins that play an important role in cell migration and ECM remodelling. Endo180 is considered to be one of these proteins. As a collagen receptor, Endo180 has the capability to regulate ligand binding and collagen uptake (East et al., 2003, Engelholm et al., 2003, Wienke et al., 2003, Kjøller et al., 2004, Thomas et al., 2005). Overexpression of Endo180 is shown to be able to promote cell migration, while silencing of Endo180 expression can inhibit cell migration (Sturge et al., 2003). As described in Section 2.4.5.2, Endo180 can promote MLC2 phosphorylation by ROCK, following upstream activation of Rho (Figure 2.5). The phosphorylation of MLC2 can generate contractile signals to promote adhesion disassembly of tumour cells (Sturge et al., 2006). Endo180 has also been shown to cooperate with uPA-uPAR in tumour cell migration and stromal collagen remodelling (Behrendt et al., 2000, Sturge et al., 2003, Kogianni et al., 2009). It is also suggested that Endo180 could work as a novel regulator of MT1-MMP activity, MT1-MMP-dependent MMP-2 activation, and uPA activity (Messaritou et al., 2009).

The ability of Endo180 to generate contractile signals that promote the disassembly of cell – cell junctions in MCF-7 cells indicates that Endo180 have a correlation with EMT during tumour progression (Sturge et al., 2006). In addition, Endo180 is expressed by invasive human breast tumours and is up-regulated by the EMT-inducing factor TGF-β in MCF-7 cells (Wienke et al., 2007). In agreement with a role in EMT progression, Endo180 is highly expressed in cells with an EMT-like phenotype and is correlated

with clinical grade in prostate cancer (Kogianni et al., 2009). However, in a recent study, an EMT suppressor role for Endo180 in complex with CD147 has been discovered in a 3D prostate epithelial cell model, which suggests that Endo180 switches its function at the earliest stages of tumour progression to become a promoter of cell invasion (Rodriguez-Teja et al., 2015b). Taken together, these findings suggest that Endo180 can act as both a promoter and suppressor of EMT. A similar dual role in the promotion or suppression of EMT has been well defined for TGF-β (Thiery, 2002). Furthermore, the pro-invasive role of Endo180 triggered by increased ECM stiffness is associated with a loss of E-cadherin and increased expression of the proteases uPA and MT1-MMP (Rodriguez-Teja et al., 2015a). All these data suggest that Endo180 is closely associated with the early stage of EMT in tumour progression and metastasis, making it a potential therapeutic target for anti-tumour therapy.

2.4.6.2 Endo180 in primary tumour growth and early lesion development

As described in the previous section, the mechanistic role of Endo180 in EMT has mostly been studied using *in vitro* models. To date, there have been two *in vivo* studies performed to investigate the contribution of Endo180 to tumour growth and progression (Curino et al., 2005, Wienke et al., 2007). Curino et al. (2005) crossed the partial Endo180 knockout mice that had been generated in previous studies with the MMTV-PyVT transgenic mice that were predisposed to malignant mammary adenocarcinoma, and reported a reduction in tumour growth compared to mice with full-length Endo180 expression (East et al., 2003, Engelholm et al., 2003). Wienke et al. (2007) conducted a mouse xenograft study, where they found that the expression of wild-type Endo180 in the human breast cancer cell line MCF-7, but not an internalisation-defective Endo180 mutant, enhanced the growth of tumours in inoculated mice. In the same study, it was also observed that areas within human tumour biopsies with high levels of

Endo180 expression had lower levels of E-cadherin compared to areas with low Endo180 expression. These results consolidate the association of Endo180 expression with EMT, and suggest that the intercellular localisation of Endo180 to endosomes is necessary for the promotion of EMT, tumour growth and tumour progression. This notion is further supported by the finding that MCF-7 cells that express the internalisation-defective Endo180 mutant display a defect in contractile signalling, cell adhesion and cell migration (Sturge et al., 2003, Sturge et al., 2006).

The inverse role of Endo180 as an EMT suppressor is evident from the finding that early lesions were observed in the prostate gland epithelium of the partial Endo180 knock-out mouse (Rodriguez-Teja et al., 2015a). It is hypothesised that these lesions are the result of increased mechanotransduction mediated by constitutive exposure of the CTLD-2 domain of Endo180, allowing it to bind to glycated collagen IV in the BM, and induce invasive behaviour in the epithelial cells.

2.4.6.3 Endo180 and bone metastasis / primary bone cancer

Immunohistochemical analysis of core biopsies of bone metastases from advanced breast cancer patients has revealed higher levels of Endo180 expression at the tumour-stromal interface than in cells within the non-tumour-adjacent bone stroma (Caley et al., 2012). Direct and indirect co-culture of prostate tumour cells and primary hOBs induced the increased expression of Endo180 in the tumour cells, associated with their participation in collagen degradation (Caley et al., 2012). A concomitant decrease in the expression of Endo180 in hOBs was associated with a decrease in their deposition of mineralised collagen matrix. These cell-type specific changes in Endo180 expression and function were found to be linked to its divergent regulation in tumour cells and osteoblasts due to dysregulated TGF-β_1 signalling (Caley et al., 2012). In a subsequent study, it was demonstrated that Endo180 was associated with the aggressive rounded

"amoeboid-like" mode of tumour cell migration on hOB-derived mineralised ECM (Caley et al., 2016). In the same study, it was shown that Endo180-dependent tumour cell migration on human fibroblast-derived ECM required LOX-dependent collagen crosslinking and stiffness of the synthesised ECM. As reported by Palmieri et al. (2013), plasma Endo180 is a promising biomarker for metastatic cancer, the increased level of which in advanced breast cancer patients is significantly decreased in patients receiving bisphosphonate treatment for bone metastasis. Results of antibody blockade experiments conducted in a mouse model of primary bone cancer (osteosarcoma) have suggested that Endo180 is a promising new therapeutic target in bone related diseases (Engelholm et al., 2016, Sturge, 2016). These previous findings form the basis of this thesis where the role of Endo180 as a biomarker and target of bisphosphonates in advanced breast cancer has been explored using an ELISA that was developed to facilitate this study.

2.5 Bisphosphonates

2.5.1 Overview

Bisphosphonates are a class of drug that can prevent the loss of bone tissue in skeletal disorders like osteoporosis, a range of bone associated cancers and bone metastases originating from solid tumours (Lin, 1996). The name of bisphosphonates comes from the two phosphonate groups in these drugs and their structural similarity to pyrophosphate. Bisphosphonates bind to mineralised bone and achieve therapeutic concentrations at sites where bone metabolism is most active (Drake et al., 2008). They are released during bone resorption and internalised by osteoclasts, then inhibit the degradation of bone by triggering the apoptosis of osteoclasts, thereby slowing bone loss (Fleisch, 2001). Bisphosphonates can be classified into two categories: nitrogenous-containing bisphosphonates (N-BPs) and non-nitrogenous

bisphosphonates. They have different mechanisms of blocking the activity and survival of osteoclasts (Green, 2004).

Amongst the non-nitrogenous bisphosphonates are clodronate, etidronate and tiludronate (McClung, 2006). These compunds achieve their pharmacological effects by replacing the terminal pyrophosphate moiety of adenosine triphosphate (ATP), forming a non-functional molecule that competes with ATP in cellular energy metabolism and stimulating apoptosis of osteoclasts to inhibit bone degradation (Frith et al., 1997).

N-BPs include alendronate, ibandronate, neridronate, olpadronate, pamidronate, risedronate and zoledronate (McClung, 2006). N-BPs act on bone metabolism by binding and blocking farnesyl diphosphate synthase in the HMG-CoA reductase pathway (mevalonate pathway). This prevents the formation of farnesol and geranylgeraniol that are essential for protein prenylation and proper sub-cellular protein trafficking (van Beek et al., 1999, van Beek et al., 2003). As a result, the prenylation of guanosine triphosphate (GTP)-binding proteins, including Ras, Rho, and Rac, is inhibited, which influences normal cellular function: including cell proliferation, cell survival and cytoskeletal dynamics (Luckman et al., 1998). In addition, inhibition of Ras signalling also dysregulates the intracellular vesicle transport in osteoclasts (Alakangas et al., 2002). Besides the signalling pathways modulated by small GTPases, N-BPs could also affect the RANKL/OPG axis and TGF-β signalling pathway (Green and Clézardin, 2010). These effects of N-BPs help to suppress the osteoclast-dependent bone degradation. It has also been shown that zoledronic acid inhibits integrin-$\alpha_V\beta_3$/Ras-dependent adhesion of the breast cancer cell line MDA-MB-231 to denatured collagen (gelatin) or vitronectin, suggesting that the anti-metastatic effect of bisphosphonates in breast cancer involves the inhibition of specific cell-ECM

interactions (Wilke et al., 2014). Based on these findings and knowledge that Endo180 is required for cell-ECM / cell-cell adhesion, it could be postulated that Endo180-dependent cell adhesion is a novel target of N-BPs (Sturge et al., 2006, Rodriguez-Teja et al., 2015b).

2.5.2 The anti-tumour effect of bisphosphonates

2.5.2.1 In vitro studies

Bisphosphonates can inhibit the proliferation and induce apoptosis in breast cancer, prostate cancer, and osteosarcoma cell lines. The biological effect of bisphosphonates in a range of tumour and bone stromal cell types mostly occurs within the concentration range of 1 μM to 100 μM (Table 2.3). Zoledronic acid is effective between 10 μM – 100 μM in supressing the proliferation and survival of MDA-MB-231 and MCF-7 cells, and the reversal of this apoptotic effect in the same cell lines by geranylgeraniol suggests that the inhibition of prenylation could be a mechanism for bisphosphonate-dependent tumour cell apoptosis (Senaratne et al., 2000, Jagdev et al., 2001). This zoledronic acid-induced apoptotic effect of MDA-MB-231 and MCF-7 cells was shown to be associated with impaired membrane localisation of Ras (Senaratne et al., 2002).

Another mechanism underlying the anti-tumour and anti-metastatic effects of bisphosphonates is their ability to prevent tumour cells from binding to the ECM, as reported in a number of earlier studies and highlighted in Section 2.5.1 (van der Pluijm et al., 1996, Boissier et al., 1997, Magnetto et al., 1999, Boissier et al., 2000, Wilke et al., 2014). The anti-invasive properties of bisphosphonates are associated with their ability to inhibit the activities of MMPs, including MMP-1, -2, -3, -7, -8, -9, -12, -13, and -14 in osteosarcoma cells, and MMP-2, -9, -12 in breast cancer cells (Teronen et al., 1999, Boissier et al., 2000, Heikkilä et al., 2002, Senaratne and Colston, 2002, Cheng et al., 2004).

Table 2.3 Effective concentrations of bisphosphonates revealed in previous studies

BPs type	*Cell line & research topic*	*Effective Concentration (μM)*	*Reference*
ALE/ETI/ IBA/PAM	Breast cancer cells, *adhesion to bone matrices*	10 – 100	van der Pluijm et al. (1996)
ZOL	Multiple myeloma cell, *cytoreductive effects*	25, 50, 100	Aparicio et al. (1998)
ZOL	Myeloma cell, *cell growth and secretion of IL-6 and MMP-1*	10, 50, 100, 500	Derenne et al. (1999)
ZOL	Breast cancer cell, *apoptosis*	10, 100	Jagdev et al. (2001)
ZOL	Prostate cancer cell, *cell growth*	25, 50, 100	Lee et al. (2001)
CLO	Osteosarcoma, *cell growth and OPG gene expression*	10 – 10000	Mackie et al. (2001)
PAM	Osteosarcoma cell, *cell growth*	10 – 50	Sonnemann et al. (2001)
ALE	Osteoclasts, *vesicular trafficking*	0.1 – 1000	Alakangas et al. (2002)
ZOL	Breast cancer cell, *membrane localisation of Ras and cytochrome c release*	50, 100	Senaratne et al. (2002)
ZOL	Pancreatic cancer cells, *antiproliferative and apoptotic effects*	1 – 100	Tassone et al. (2003)
ALE/ETI/ PAM/RIS	Osteoclasts, *antiresorptive action*	10 – 100	Van Beek et al. (2003)
ZOL	Osteoblast-like cell, *RANKL expression*	5	Pan et al. (2004)
ALE/ZOL/ CLO/IBA/	Breast cancer cell, *anti-tumour effects*	10	Verdijk et al. (2007)
ALE	Osteoblast-like cell, *proliferation and osteogenic differentiation*	0.001, 0.1, 10	Xiong et al. (2009)
ALE	Prostate cancer cell, *PKC inhibition*	100	Tatsuda et al. (2010)
ZOL	Prostate cancer cell, *expression profile of WNT molecules*	10 – 1000	Thiele et al. (2011)
ZOL	Breast cancer cell, *$\alpha_v\beta_3$-mediated adhesion*	1 – 100	Wilke et al. (2014)
ZOL	Breast cancer cell, *differential anti-tumour effects*	50	Wilson et al. (2015)

Note:
BPs – bisphosphonates, ALE – alendronic acid, ZOL – zoledronic acid, CLO – clodronate, ETI – etidronate, IBA – ibandronate, PAM – pamidronate, RIS – risedronate, IL-6 – Interleukin 6, MMP-1 – matrix metalloproteinase-1, OPG – Osteoprotegerin, RANKL - Receptor activator of NF-κB ligand, WNT - Wingless-type MMTV integration site family protein

The effect of bisphosphonates on uPA activity appears to be cell type-dependent, with no effect observed in osteosarcoma cell lines, but inhibition in the prostate cancer cell line PC-3 due to inhibition of PKC mechanism, both of which play crucial roles in cancer progression (Teronen et al., 1999, Tatsuda et al., 2010). Given the current knowledge about molecular interactions of Endo180 (Figure 2.5), which include uPA, PKC, CD147 and MMPs, it is possible that the suppression of its shedding by bisphosphonates in advanced breast cancer patients involves inhibition of the extracellular matrix metalloproteinase inducer CD147, and the activities of PKC, uPA and MMPs, leading to decreased cell-ECM adhesion, cell migration and invasion (Isacke et al., 1990, Behrendt et al., 2000, Sturge et al., 2003, Messaritou et al., 2009, Palmieri et al., 2013a, Rodriguez-Teja et al., 2015b).

2.5.2.2 In vivo studies

There are also a number of *in vivo* studies that have demonstrated the protective effect of bisphosphonates in metastatic bone disease. In two animal models, zoledronic acid was shown to halt existing bone metastases from progressing and prevent new metastases from developing (Green et al., 2000, Peyruchaud et al., 2001). Given the fact that bisphosphonates can reduce osteogenesis and bone degradation by inhibiting osteoclast activity, the mechanism of its anti-tumour effects might be also based on this. The reduction of bone degradation can cause a reduction of growth factors that are needed by tumour cells to invade bone tissue.

In a recent study the survival benefit of bisphosphonates uncovered in clinical trials (Section 2.5.2.3) were studied in models that mimic pre- and postmenopausal status and allow for investigation of their effect on altered bone turnover and growth of disseminated breast tumour cells (Ottewell et al., 2014). It was found that ovariectomised mice mimicking the postmenopausal status displayed increased growth

of disseminated tumour cells in bone, that was completely prevented by 4× weekly injections of 100 mg/kg zoledronic acid. In contrast, the same zoledronic acid treatment regimen had no effect on tumour growth in control mice that had undergone sham operations. The results of these experiments are the first to demonstrate that tumour growth is driven by osteoclast-mediated mechanisms in models that mimic post- but not premenopausal bone, explaining the divergent antitumor effects of bisphosphonate reported in these settings.

2.5.2.3 Clinical studies

A survival benefit in breast cancer patients receiving bisphosphonates has been reported in a number of clinical trials (Gnant et al., 2009, Coleman et al., 2011, Gnant et al., 2011). Meta-analyses of individual patient data from multiple clinical trials has confirmed that adjuvant bisphosphonate treatment in early breast cancer patients can reduce the rate of breast cancer recurrence in the bone and improve breast cancer survival in postmenopausal female breast cancer patients (Early Breast Cancer Trialists' Collaborative Group, 2015). These findings have been used to establish new guidelines on personalised treatment of breast cancer patients with bisphosphonates (Hadji et al., 2016). The efficacy of bisphosphonates in this clinical setting strongly suggests that their anti-tumour effect require further investigation and this may lead to the identification of new targets in metastatic cancer, which may include the regulatory control of Endo180 function, its interaction partners, and its downstream signalling pathways, as mention above and in Section 2.4.6.

2.6 Matrix metalloproteinase (MMP)

2.6.1 Overview

MMPs are a family of endopeptidases that are dependent on Ca^{2+} and Zn^{2+} metal ions as cofactors (Figure 2.6). The first MMP (MMP-1) was identified following the

observation that tadpole tails secreted a factor that could degrade fibrillar collagen (Gross and Lapiere, 1962). Six years later, the human form of this collagenase was isolated from human skin (Eisen et al., 1968). Later studies successively revealed that MMPs are synthesised in a latent form (zymogen) that required cleavage of their pro-peptide to become activated (Harper et al., 1971, van Wart and Birkedal-Hansen, 1990).

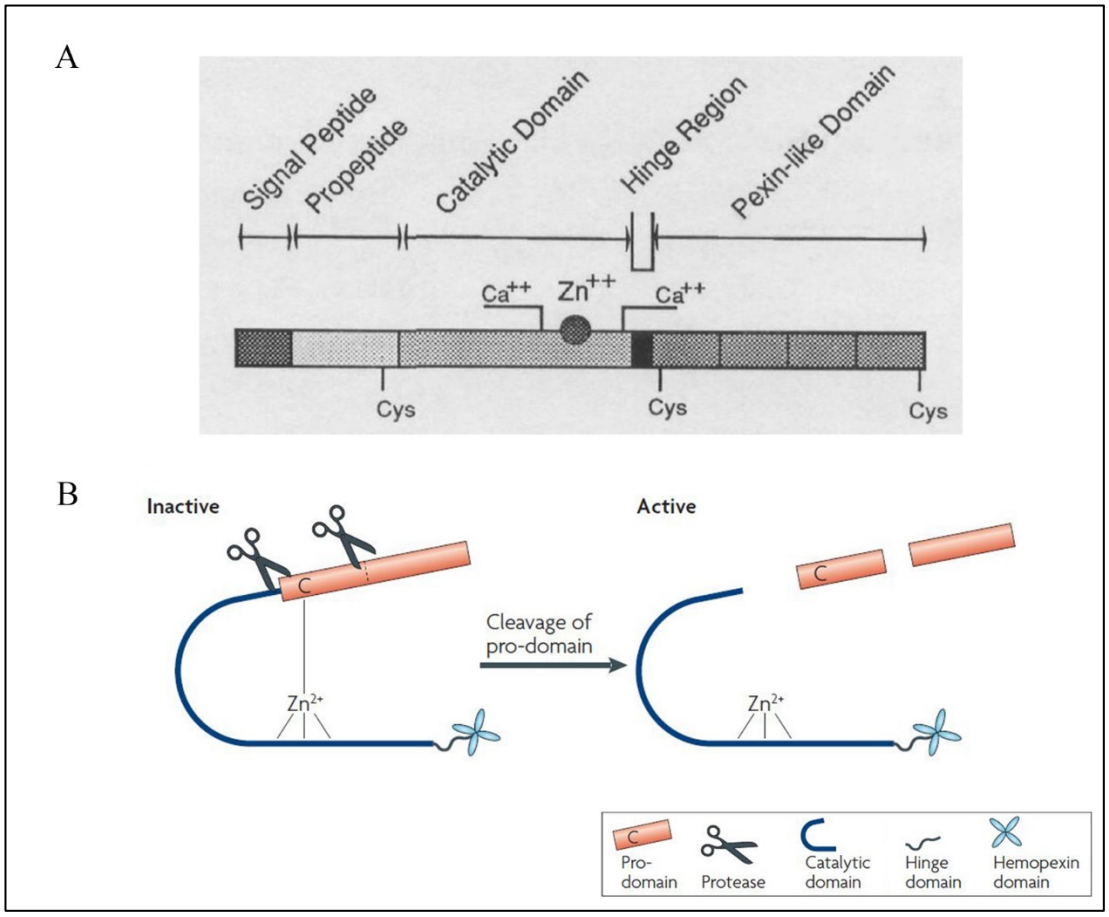


Figure 2.6 Structure and activation of MMPs

A) MMPs contain five domains: a 17–29 residue hydrophobic signal peptide, a 77–87 residue propeptide, a catalytic domain that contains the catalytic site, a 5–50 residue hinge region, and a ~200 residue hemopexin or vitronectin-like C-terminal domain. B) A conserved cysteine residue (Cys) in the pro-domain coordinates the zinc ion, which would otherwise be used for catalysis. The pro-domain is removed by a combination of a cleavage in the domain and a cleavage between the pro-domain and the catalytic domain. Adapted from Birkedal-Hansen et al. (1993) and Page-McCaw et al. (2007) with permissions.

As is shown in Figure 2.6, the family members of MMPs have a common domain structure (Birkedal-Hansen et al., 1993, Page-McCaw et al., 2007): 1) The hydrophobic signal peptide domain is followed by the pro-peptide domain, the main role of which is

to maintain the stability of the zymogen. Once the pro-peptide domain is removed, the enzyme will be activated to bind and cleave its substrate. 2) The catalytic domain contains a Zn^{2+} binding motif that is important for its enzymatic activity. 3) The flexible hinge which is rich in proline connects to the C-terminal pexin-like/hemopexin domain, which functions to determine substrate specificity. 4) Among these domains, the pro-peptide domain and catalytic domain are highly conserved between family members, while slight differences are observed in other domains.

The main role of MMPs is considered as endopeptidases that catalyse the degradation of the ECM (Birkedal-Hansen et al., 1993). Each MMP has its own substrate specificity, but this is not absolute: one MMP can catalyse the degradation of several different components of the ECM, while a certain ECM component can be degraded by various MMPs, while the catalytic efficiency might be different (Birkedal-Hansen et al., 1993).

With the progress of further studies, MMPs are now known to have more complex functions than only degrading the ECM, as they are believed to be able to affect cell behaviours by regulating the activity of ECM proteins and signalling molecules (Streuli, 1999, Sternlicht and Werb, 2001). MMPs, together with other proteinase families such as ADAM (a disintegrin and metalloproteinase) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs), can mediate the cleavage of cell membrane proteins, including those involved in EMT, such as E-cadherin, CD44 and TGF-α/β (Yu and Stamenkovic, 2000, Kajita et al., 2001, Noe et al., 2001, Edwards et al., 2008).

2.6.2 MMPs and bone turnover

2.6.2.1 Overview

MMPs play a vital role in bone development and pathology (Rodríguez et al., 2010). MMP gene knockout mice and experimental bone disease models have revealed that

MMP-2, MMP-9, MMP-13, MT1-MMP and MT3-MMP are the MMP family members with the most critical roles during skeletal development (Paiva and Granjeiro, 2014). To date, the partial Endo180 knockout mouse has been crossed with both MMP-2 and MT1-MMP knockout mice and shown to have a number of cooperative and distinct functions in bone development and homeostasis (Wagenaar-Miller et al., 2007, Madsen et al., 2013). The original observation that Endo180 was co-expressed with MMP-13 during osteogenesis (Engelholm et al., 2001b) led to consideration that they might be interaction partners, but further study found that there was no strong evidence for this interaction (Bailey et al., 2002).

2.6.2.2 The role of MMP-2 in bone turnover

MMP-2 is a 72 kDa gelatinase that can cleave collagens type I, IV, V, VII and XI, gelatin, fibronectin, laminin, elastin, aggrecans and large tenascin-C (Shimokawa Ki et al., 2002). MMP-2 knockout mice display a subtle delay in growth, and have osteopenic long bones that are prone to spontaneous fracture (Itoh et al., 1997, Inoue et al., 2006). This defect in MMP-2 knockout mice is correlated with a significant decrease in mineralisation density and mineral-to-collagen ratio (Nyman et al., 2011). The progressive loss of bone mineral density, articular cartilage destruction and abnormal long bone and craniofacial development observed in MMP-2 knockout mice were found to be associated with decreases in osteoblast and osteoclast numbers *in vivo*; and in accordance, the targeted inhibition of MMP-2 in human and murine osteoblast cell lines resulted in a decrease in their proliferation rates *in vitro* (Mosig et al., 2007). Based on these facts, it is believed that the role of MMP-2 in bone development is to regulate osteoblast and osteoclast activities and proliferation, although the molecular mechanisms responsible for these effects are still not known.

2.6.2.3 The role of MMP-9 in bone turnover

MMP-9 is a 92 kDa gelatinase that cleaves collagens type IV, V and XI, gelatin and elastin in the ECM (Shimokawa Ki et al., 2002). In early development, MMP-9 is expressed in trophoblasts and osteoclasts, suggesting that it plays a role in embryo implantation and bone resorption (Reponen et al., 1994, Alexander et al., 1996). During adulthood, MMP-9 is mainly expressed in inflammatory cells associated with different types of bone pathology and cancer (Corbel et al., 2000, Parks et al., 2004). The fundamental role of MMP-9 as an essential regulator of growth plate angiogenesis and the apoptosis of hypertrophic chondrocytes was identified in a mouse model; and galectin-3 was identified as the MMP-9 substrate with an important functional role in this stage of bone development (Vu et al., 1998, Colnot et al., 2001, Ortega et al., 2005, Kojima et al., 2013). This is supported by the finding that the transplant of wildtype bone marrow into MMP-9 knockout mice can correct these bone growth defects of abnormal growth plate development and delayed hypertrophic cartilage apoptosis (Vu et al., 1998). It has also been noted that MMP-9 knockout mice have brittle bones with less dense and highly disordered trabeculae, a phenotype that is possibly due to a subsequent defect in the organization of collagen and other ECM components in the bone matrix (Nyman et al., 2011).

2.6.2.4 The role of MT1-MMP in bone turnover

MT1-MMP is a highly proteolytic membrane-anchored enzyme expressed in skeletal tissue and cancer cells that can mediate the cleavage and activation of the secreted MMP-2 zymogen (Kinoh et al., 1996, Ohuchi et al., 1997). MT1-MMP knockout mice have serious defects in bone formation and displayed very high mortality rates (Ohuchi et al., 1997, Holmbeck et al., 1999, Zhou et al., 2000). Given that MT1-MMP is the major activator of MMP-2, it is not surprising that MT1-MMP null mice show

decreased MMP-2 activity (Strongin et al., 1995, Oblander et al., 2005). Moreover, MMP-2 and MT1-MMP double gene knockout mice die immediately after birth (Oh et al., 2004).

2.6.2.5 The cooperation of MMPs with Endo180 in bone turnover

The correlation of Endo180 and MMPs in ECM remodelling, especially collagen degradation, has also been investigated using double gene knockout mouse models. A study by Wagenaar-Miller et al. (2007) revealed the complementary *in vivo* roles of Endo180 and MT1-MMP in collagen degradation and bone development in Endo180 and MT1-MMP double gene knockout mice; these mice also displayed a marked decrease in survival compared to their single gene knockout counterparts. An aligned study by Madsen et al. (2007) demonstrated that Endo180-deficient fibroblasts from knockout mice could not mediate intracellular degradation of the collagen fragments generated through MT1-MMP/MMP-2 mediated cleavage, while wild-type fibroblasts directed an organized and complete collagen degradation sequence. These findings indicate that Endo180 and extracellular collagenases cooperate in fibroblast-mediated collagen degradation. The results of a later double gene knockout mouse study by Madsen et al. (2013) corroborated that Endo180-deficient and MMP2-deficient adult mice have shorter tibia and femurs and reduced bone mineral density and trabecular bone quality with additional decreases in long bone growth and length observed in Endo180/MMP-2 double gene knockout mice. Interestingly, the membranous bone thickening observed in MMP-2 knockout mice was counteracted in Endo180/MMP-2 double knockout mice, indicating that Endo180 and MMP-2 are not coupled to similar bone ECM remodelling processes during skull development. These findings indicate that Endo180 promotes bone ECM formation, as previously uncovered following the genetic silencing of Endo180 in hOBs, providing further evidence to the postulated

contribution of osteoblastic Endo180 upregulation to the formation of osteosclerotic lesions in bone (Sturge et al., 2011, Caley et al., 2012).

2.6.3 MMPs and tumour metastasis

MMPs can collectively degrade most protein components in the ECM, leading to breakdown of histological barriers like the BM that normally obstruct cell invasion (Kessenbrock et al., 2010). This suggests that MMPs play a role in the early stages of EMT that lead to tumour cell invasion and metastasis.

MMP-1, -2, -13 and MT1-MMP drive the ECM remodelling processes that facilitate the migration and invasion of tumour cells, and the cell surface localisation of MT1-MMP is postulated to be the most essential player in this process (Sabeh et al., 2004, Wolf et al., 2007, Friedl and Wolf, 2008). MMP-2, MMP-9 and MT1-MMP can trigger and regulate the activation of TGF-β from its latent form, which in turn can promote tumour-associated tissue remodelling and angiogenesis (Yu and Stamenkovic, 2000, Dallas et al., 2002, Mu et al., 2002, Massagué, 2008, Tatti et al., 2008).

The overexpression of MMP-3 has been shown to trigger EMT in mammary epithelial cells by promoting the cleavage of E-cadherin, downregulating cytokeratins, upregulating vimentin, inducing keratinocyte growth factor expression and activation and upregulating endogenous MMPs (Lochter et al., 1997, Sternlicht et al., 1999).

A significant finding made in a mouse model of bone metastasis, was the identification of MMP-7 expression by osteoclasts, located at the interface between prostate tumour cells and the bone stroma, as a promotor of osteolysis and bone metastasis through its activation of RANKL and subsequent promotion of osteoclast differentiation (Lynch et al., 2005). A similar mechanism has been observed in metastatic breast cancer, where MMP-1 and ADAMTS-1 activate the RANKL pathway by releasing EGF-like growth

factors from osteoblasts, leading to the promotion of osteolysis and progression of bone metastasis (Kang et al., 2003, Lu et al., 2009).

2.7 Project aims and hypotheses

2.7.1 Aims

- Develop an accurate and precise immunoassay that is suitable for wider clinical applications to measure plasma Endo180 in breast cancer patients.
- Further explore the biological and clinical implications of Endo180 as a breast cancer biomarker.
- Investigate the effect of bisphosphonates on the expression and release of Endo180 in tumour cells and osteoblasts.
- Investigate the mechanism(s) underlying the release of Endo180.

2.7.2 Hypotheses

- Endo180 is a potential biomarker for advanced breast cancer.
- Bisphosphonates modulate the expression and release of Endo180 in breast cancer cells and osteoblasts.
- MMPs modulate the release of Endo180 from breast cancer cells.
- The indirect coculture of breast cancer cells and osteoblasts affects the release of Endo180 from both cell types.

3 Materials and Methods

3.1 Materials

3.1.1 Cell culture

3.1.1.1 Media

- Dulbecco's Modified Eagle's Medium (DMEM; Gibco, 41965-062)
- Minimum Essential Medium (MEM; Gibco, 31095-052)
- Keratinocyte Serum Free Medium (K-SFM; Gibco, 17005-042)
- McCoy's 5A Modified medium (Gibco, 26600-080)
- Human Osteoblast Growth Medium (Sigma-Aldrich, 417-500)
- CD Hybridoma Medium (11279-023, Gibco)

3.1.1.2 Reagents

- Fetal bovine serum (FBS; Gibco, 10270-106)
- Phosphate buffered saline (PBS; Gibco, 14190-169)
- TrypLE™ express enzyme (1×) (Gibco, 12604-039)
- L-Glutamine (25030-081, Gibco)
- Insulin (Sigma-Aldrich, I0516)
- G418 sulfate (Geneticin, 10131-027)
- Non-essential amino acids (NEAA; Lonza, 13-114E)
- Penicillin-Streptomycin (Gibco, 15070-063)
- 70% (v/v) ethanol (VWR, 20821.330)
- Trypan blue solution, 0.4% (w/v) (Gibco, 15250-061)
- Dimethyl sulfoxide (DMSO; Fisher Scientific, 10103483)

3.1.1.3 Equipment

- Variable volume sterile micropipette & tips (Gilson)
- Sterile pipets (Sarstedt)
- Tissue culture flasks (Sarstedt)
- Cryovial (Sarstedt)
- Six-well plate (Sarstedt)
- Microcentrifuge tube (Sarstedt)
- Hemocytometer with coverslip (Marienfeld, 29811)
- Mr Frosty™ Freezing Container (Thermo Fisher Scientific, 5100-0001)
- Tally counter (Rosefame, ETC-002)
- Heraeus™ Megafuge™ 16 Centrifuge (Thermo Fisher Scientific)
- Class II Biological Safety Cabinet (ESCO)
- CO2 incubator

3.1.2 Cell lysis

- RIPA buffer: 25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% v/v NP-40, 1% w/v sodium deoxycholate, 0.1% w/v SDS (Thermo Fisher Scientific, 89900)

- Protease and phosphatase inhibitor cocktail, 1% v/v (Thermo Fisher Scientific, 78440)
- Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23227).
- Heraeus™ Fresco™ 17 Microcentrifuge (Thermo Scientific)

3.1.3 Antibody purification

- Sterile vacuum filtration unit with 0.45 µm pore size membrane (Sarstedt, 83.1822)
- HiTrap™ Protein G HP 5mL (GE Healthcare, 17-0405-01)
- Binding buffer: 20 mM sodium phosphate, pH 7.0
- Elution buffer: 0.1 M glycine-HCl, pH2.7
- Neutralisation buffer: 1 M Tris, pH 9.5
- Dialysis tube (Fisher Scientific, 15253847)
- NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific)

3.1.4 Production of Endo180 standard protein

- Pierce™ Crosslink Immunoprecipitation Kit (26147, Thermo Fisher Scientific)
- EMD Millipore Amicon™ Ultra-0.5 Centrifugal Filter Units (UFC500308, Merck Millipore)
- NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific)
- Pierce™ BCA Protein Assay Kit (23227, Thermo Fisher Scientific)
- Anti-Endo180 mAb A5/158

3.1.5 Endo180 ELISA

- Coating buffer: 0.2 M sodium carbonate/bicarbonate, pH 9.4
- Capture antibody: anti-Endo180 mAb A5/158 diluted using 1/5 strength standard diluent
- Wash buffer: 1 L 0.1 M phosphate, 0.15 M sodium chloride, pH 7.2, containing 0.05% Tween-20
- Blocking buffer / Standard diluent: 2% w/v bovine serum albumin (BSA) in wash buffer. Prepare freshly for each assay
- Detection antibody: biotinylated anti-Endo180 mAb 39.10 diluted using 1/5 strength Standard diluent
- Enzyme conjugate: HRP-conjugated streptavidin (N100, Thermo Scientific) diluted to the working concentration using 1/5 strength standard diluent
- Substrate: TMB substrate kit (34021, Thermo Scientific), two reagents should be mixed together in equal volumes within 15 minutes of use. Protect from light
- Stop solution: 2 M sulfuric acid
- 96-well microplate (Thermo Scientific, 15041)
- Adhesive strip (Thermo Scientific, 15036)

3.1.6 Patient sample preparation

- Vacutainer blood collection tube with spray-coated K2EDTA (BD, 367525)
- Screw cap microcentrifuge tubes (Thermo Fisher Scientific, 3463)

3.1.7 Drug treatment

Drugs used in this study include: two clinically used nitrogenous bisphosphonates – alendronic acid and zoledronic acid; recombinant full-length active human MMP-2 protein; broad-spectrum MMP inhibitor – Marimastat. Upon delivery, the drugs were dissolved in corresponding solvent to the stock concentration, and were stored at -20℃ as aliquots. When being used to treat the cells, the stock solution was diluted in corresponding conditioned medium for the cell line being treated. Details of the drugs are shown in Table 3.1.

Table 3.1 Drugs used to treat cells in this study

Drug	*Manufacture*	*Catalogue number*	*Solvent*	*Stock concentration*
Alendronic acid	Sigma-Aldrich	A4978	1× PBS	2 mM
Zoledronic acid	Sigma-Aldrich	SML0223	1× PBS	2 mM
MMP-2	Abcam	ab81550	H_2O	0.1 mg/mL
Marimastat	Abcam	ab141276	DMSO	1 mM

3.1.8 Western blot

- The SDS-PAGE buffer system stock solutions (*Table* 3.2)
- Discontinuous polyacrylamide gel, 7.5% (w/v) (*Table 3.3*)
- Transfer buffer: pH 8.3, 25 mM Tris, 192 mM glycine, with 20% v/v Methanol
- Blocking buffer: Tris-buffered saline (TBS) with 0.05% v/v Tween-20 and 5% w/v of non-fat dry milk
- Washing buffer: TBS with 0.05% v/v Tween-20
- Protein ladder: Precision Plus Protein™ WesternC™ Standards (Bio-Rad, 161-0376), PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, 26616)
- Primary antibody and secondary antibody
- Substrates: Enhanced chemiluminescence substrate (Thermo Fisher Scientific, 32209)
- Immobilon-P Polyvinylidene Fluoride (PVDF) Membrane (Merck Millipore, IPVH00010)
- Mini-PROTEAN Tetra System and power pack (Bio-Rad).
- Centrifuge tube, 15/50 mL (Sarstedt)

Table 3.2 SDS-PAGE buffer system stock solutions

Buffer	Reagent	Quantity	Remark
Acrylamide/Bis (30% T, 2.67% C)	Acrylamide	87.6 g	store at 4℃
	N'N'-bis-methylene-acrylamide	2.4 g	
	Deionized water (ddH_2O)	300 mL	
10% w/v SDS	SDS	10 g	
	ddH_2O	100 mL	
Resolving Gel Buffer	Tris base	27.23 g	Adjust to pH 8.8 with HCl
	ddH_2O	150 mL	
Stacking Gel Buffer	Tris base	6 g	Adjust to pH 6.8 with HCl
	ddH_2O	100 mL	
Sample Buffer (SDS reducing buffer)	0.5 M Tris-HCl, pH 6.8	1.25 mL	
	Glycerol	2.5 mL	
	10% w/v SDS	2.0 mL	
	0.5% w/v Bromophenol Blue	0.2 mL	
	ddH_2O	3.55 mL	
10x Running Buffer	Tris base	30.3 g	
	Glycine	144 g	
	SDS	10 g	
	ddH_2O	1,000 mL	
10% w/v APS	Ammonium persulfate	100 mg	Prepare fresh
	ddH_2O	1 mL	

Table 3.3 Formulation for 7.5% (w/v) discontinuous polyacrylamide gel

Gel (10mL)	ddH_2O (mL)	Acrylamide/Bis (mL)	Resolving/Stacking Gel Buffer (mL)	10% SDS (mL)	10% APS (µL)	TEMED (µL)
Stacking Gel	6.1	1.3	2.5	0.1	50	10
Resolving Gel	5.1	2.3	2.5	0.1	50	5

**APS & TEMED was added immediately prior to pouring the gel*

3.1.9 Flow cytometry

- Sterile 1× DPBS (Gibco®, 14190-169)
- Blocking buffer: 5% w/v BSA (Sigma-Aldrich, A2153)
- Fixation buffer: 4% w/v PFA (Insight Biotechnology, sc-281692)
- Primary antibody and secondary antibody
- Flow cytometry tubes (Sarstedt)
- Microcentrifuge tube (Sarstedt)
- Heraeus™ Fresco™ 17 Microcentrifuge (Thermo Scientific)
- FACSCalibur™ Flow cytometer (BD Biosciences, San Jose, CA).

3.1.10 Apoptosis assay

- APO-BrdU™ TUNEL Assay Kit with Alexa Fluor® 488 Anti-BrdU (A23210, ThermoFisher Scientific)

3.1.11 Gelatin zymography

- 4× Sample buffer: 250 mM Tris-HCl, pH 6.8; 40% v/v glycerol; 8% w/v SDS; and 0.01% w/v bromophenol blue

- Renaturing solution: 2.5% v/v Triton X-100 in dH_2O
- Developing buffer: 0.2 M NaCl, 5 mM CaCl2, 0.02% v/v Brij 35, 50 mM Tris–HCl, pH 7.8
- Staining solution: 0.2% w/v Coomassie brilliant blue
- Destaining solution:10% v/v methanol, 5% v/v acetic acid in dH_2O).
- Precast polyacrylamide gels containing 0.1% w/v gelatin (161-1167, Bio-Rad)

3.1.12 Statistical analysis and power calculation

- SPSS statistics, version 24.0 (IBM Corporation)

3.2 Methods

3.2.1 Cell culture

3.2.1.1 Cell lines used in the study

Human breast adenocarcinoma cell line MDA-MB-231 (cat. 92020424) and MCF-7 (cat. 86012803), human prostate adenocarcinoma PC-3 (cat. 90112714), human osteosarcoma cell line U-2 OS (cat. 92022711) were obtained from the European Collection of Authenticated Cell Cultures (ECACC, Public Health England, Salisbury, UK). The human osteoblast cell line hOB (cat. C-12720) was obtained from PromoCell GmbHS (Heidelberg, Germany). Human prostate epithelial cell line RWPE-1 (cat. CRL-11609) was obtained from the American Type Culture Collection (ATCC, LGC Standards, Teddington, UK).

The establishment of Endo180 overexpressing cells has been previously reported (Sheikh et al., 2000, Howard and Isacke, 2002, Wienke et al., 2003). Briefly, the pcDNA3 vector that was inserted with full-length cDNA of the Endo180 coding region was transfected into MCF-7 cells to enable Endo180 expression. Stable transfectant that expresses Endo180 was screened by G418 resistance and named as MCF7-E cell line. Similarly, MCF-7 cells that were transfected to express mutated non-endocytic Endo180 were established and named as MCF7-D cell line. The MCF7-E and MCF7-

D cell lines were established previous to this study and were preserved in liquid nitrogen.

All cells used in this study were regularly tested to be mycoplasma free.

3.2.1.2 Cell culture media preparation

The MDA-MB-231, MCF-7 and PC-3 cells were cultured in DMEM containing 10% FBS. MCF7-E/D cells were cultured in MEM that contains 10% FBS, 10 µg/mL Insulin, 0.5 mg/mL G418 Sulfate, and 1% NEAA. U-2 OS cells were cultured in McCoy's 5A Modified medium with 10% FBS. RWPE-1 cells were cultured in K-SFM containing bovine pituitary extract and human recombinant epidermal growth factor. hOBs were cultured in human osteoblast growth medium. Penicillin-Streptomycin were added to all cell culture media to a final concentration of 50 units/ml penicillin and 50 µg/ml streptomycin.

3.2.1.3 General cell culture procedures

All cell culture procedures were carried out in the Class II Biological Safety Cabinet unless otherwise indicated. All cell flasks, reagent containers and equipment were sprayed with 70% ethanol before being moved into the cabinet.

Culture of all cell lines was started from frozen cells. Once the cell-containing cryovials were collected from liquid nitrogen, they were immediately placed in 37℃ water bath with constant agitation for 1 – 2 minutes. Cells were then washed with fresh pre-warmed medium to remove DMSO, centrifuged, re-suspended in a T25 cell culture flask that contained pre-warmed medium. The flask was then put into in a cell incubator and kept at 37℃ in a humidified atmosphere and 5% CO_2. Cells were checked after 24 hours under an inverted microscope and medium was refreshed regularly.

Cells were sub-cultured once become confluent. Spent medium was discarded and cell monolayer was washed with PBS to remove serum. TrypLE™ Express Enzyme was used to detach cells. Serum-containing medium was then added to neutralize the enzyme. Hemocytometer was used to determine the cell density, and cells were split into new cell culture flasks at 2×10^5 cells/mL.

After the study finished, cells were frozen for long-term preservation. Cells in logarithmic growth phase were washed with PBS, trypsinised and centrifuged. The number of cells were measured by mixing equal amounts of cell suspension with 0.4% (w/v) trypan blue solution, counted using a hemocytometer under a microscope. Cells were then re-suspended with freezing media – corresponding cell growth medium that contains 20% FBS and 5% DMSO, at a concentration of 1×10^7 cells/mL. Cell-containing freezing media were added into each cryovial. Cryovials were put into a freezing container and placed in -80℃ freezer overnight, and transferred to liquid nitrogen for long-term preservation.

3.2.2 Indirect co-culture

MDA-MB-231, MCF-7, MCF7-E, PC-3, hOB cells were recovered and cultured as described in Section 3.2.1.

To collect conditioned medium for indirect co-culture, MDA-MB-231, MCF7-E and hOBs were plated in T-175 flasks and cultured to 90% confluence respectively. The medium in each flask was then replaced with 30 ml of corresponding fresh growth medium. After 48 hours, the conditioned medium was removed, centrifuged at 4,000 rpm for 10 min, aliquoted and frozen at -20℃ until use.

To study the effect of indirect contact between tumour cells (MDA-MB-231 and MCF7-E) and osteoblastic cells (hOB), five different experimental conditions were established:

1) MDA-MB-231 in the conditioned medium of hOB: MDA-MB-231 cells were seeded in a 6-well plate at 2×10^5 cells/well and cultured in fresh growth medium for 24 hours, and were then washed with PBS for three times before adding the conditioned medium obtained from hOB. Cells were then cultured at 37℃ for 48 hours before the conditioned medium and the cells were collected and applied to further analysis.

2) MCF7-E in the conditioned medium of hOB: MCF7-E cells were seeded in a 6-well plate at 2×10^5 cells/well and cultured in fresh growth medium for 24 hours, and were then washed with PBS for three times before adding the conditioned medium obtained from hOB. Cells were then cultured at 37℃ for 48 hours before the conditioned medium and the cells were collected and applied to further analysis.

3) hOB in the conditioned medium of MDA-MB-231: hOB cells were seeded in a 6-well plate at 2×10^5 cells/well and cultured in fresh growth medium for 24 hours, and were then washed with PBS for three times before adding the conditioned medium obtained from MDA-MB-231. Cells were then cultured at 37℃ for 48 hours before the conditioned medium and the cells were collected and applied to further analysis.

4) hOB in the conditioned medium of MCF7-E: hOB cells were seeded in a 6-well plate at 2×10^5 cells/well and cultured in fresh growth medium for 24 hours, and were then washed with PBS for three times before adding the conditioned medium obtained from MCF7-E. Cells were then cultured at 37℃ for 48 hours before the conditioned medium and the cells were collected and applied to further analysis.

5) Control: under the control condition, MDA-MB-231, MCF7-E and hOB cells were seeded at 2×10^5 cells/well in each 6-well plate and cultured as described above in each

co-culture experiment, except during the medium change procedures, fresh growth medium for the cell line from which the conditioned medium was collected were added, instead of the conditioned medium.

3.2.3 Cell lysis

Following the procedures in Section 3.1.3, after the final wash with PBS, 1× RIPA buffer was added to the microcentrifuge tube that contained the cells collected from each well to re-suspend the cells. The mixture of pellet and lyse buffer were put on ice for 15 minutes and then operated by sonication for 5 minutes (5 cycles with 30-second break) to break DNA into fragments and reduce solution viscosity. Then the tubes were centrifuged at 4℃, 20,000 ×g for 10 minutes. Pellets were discarded and the supernatant that contained protein was transferred into fresh microcentrifuge tubes. The concentration of protein in cell lysate was measured using BCA Protein Assay Kit according to the manufacturer's manual. The samples were stored at -20 ℃ before further analyses.

3.2.4 Antibody production and purification

3.2.4.1 Antibody production

The anti-Endo180 monoclonal antibodies, A5/158 and 39.10, were produced by two strains of hybridomas. These hybridomas were generated by immunised mice that were immunised with AG1523 human fibroblasts and purified full-length human Endo180 respectively, the procedure of which has been previously reported (Isacke et al., 1990, Sheikh et al., 2000, Wienke et al., 2007). The hybridomas were generated before the start of this study and were preserved in liquid nitrogen for future use.

In this study, the hybridomas in cryovials were removed from liquid nitrogen and rapidly thawed in 37℃ water bath, and then transferred into a T75 tissue culture flask containing 20 mL pre-warmed fresh CD Hybridoma Medium containing 8 mM L-

Glutamine. No centrifuge was performed at this stage in order to prevent damage to hybridomas. Cells in tissue culture flasks were incubated at 37℃ in a humidified atmosphere of 5% CO_2 in air. After 24 hours, medium was changed and cells were transferred into T175 tissue culture flasks with pre-warmed media. Cells were sub-cultured in logarithmic growth phase 4 – 5 days after transfer with a seeding density of 2×10^5 cells/mL, and then sub-cultured once viable cell density reaches 1×10^6 cells/mL (usually 3 days after previous split). Spent media containing antibody production was collected and stored in -20℃ for before filtration and purification.

After enough media has been collected, hybridomas were frozen in logarithmic growth phase with the viability ≥ 90% and cell density of 1×10^7 cells/mL. Freezing media for hybridomas contains 90% CD Hybridoma Media and 10% DMSO, and was pre-cooled to 4℃ before use.

3.2.4.2 Antibody purification and verification

Media collected from hybridoma culture were first filtered using a sterile vacuum filtration unit with 0.45 μm pore size membrane to remove particulate ingredient. HiTrap™ Protein G HP was used to purify antibodies from the medium. The column was washed with binding buffer at 5 mL/min before antibody-containing media were applied via a syringe fitted to the luer connector at 5 mL/min. The column was then washed again with binding buffer, after which elution buffer were added to elute the antibody. Eluted antibody-containing buffer was collected into collection tubes that contained neutralisation buffer. The antibody in elution buffer were then applied to overnight buffer exchange with PBS using a piece of dialysis tube. The purified antibodies were validated in SDS-PAGE to check the purity. Measurement of the concentrations of each antibody was performed using NanoDrop 2000

spectrophotometer. Aliquots of validated purified antibodies were kept in -20℃ for long-term storage.

3.2.5 Production of Endo180 standard protein

3.2.5.1 Cell culture and protein expression

MCF7-E cells were recovered and cultured in T175 cell culture flasks as described in Section 3.2.1. Cells were allowed to expand and reach 95% confluent before being collected into microcentrifuge tubes. Cells were lysed as described in Section 3.2.3. Cell lysates were and then pre-cleared using the control agarose resin before being applied to immunoprecipitation procedures.

3.2.5.2 Immunoprecipitation

Crosslink immunoprecipitation was carried out according to the manufacturer's manual with small optimisations to scale up the processing capacity. The protein A/G plus agarose was added into each spin column and washed twice with 1× coupling buffer. Antibody solution was adjusted to 0.5 μg/μL in 1× coupling buffer and was added to each column. The column with added antibodies was put on a rotator and incubated for 1 hour at room temperature, and then washed with 1× coupling buffer three times. DSS in DMSO (2.5 mM) was adjusted to working concentration (450 μM) using 1× coupling buffer and was added to each column, followed by 1-hour incubation to crosslink the bound antibody. The column was then washed twice with elution buffer to quench the reaction and remove non-crosslinked antibody. The column was then washed twice with cold lysis/wash buffer before adding samples. Half the volume of the pre-cleared cell lysate collected from one T175 flasks was added into each column, and then incubated overnight at 4℃ with end-over-end mixing. The column was then centrifuged and washed with 1× TBS three times and of 1× conditioning buffer once. Before the elution step, neutralisation buffer (1 M Tris, pH 9.5) was added to each

collection tube to neutralise the eluent that contains Endo180. Elution buffer was added into the column and centrifuged immediately, followed by adding elution buffer and incubating for 5 minutes before the final centrifugation. The elution step was repeated twice and each eluate was analysed separately to ensure that the antigen has completely eluted. The column was washed twice with of 1× coupling buffer, and filled with 1× coupling buffer to preserve the activity of antibody-coupled resin for short-term storage. Eluted protein and washed off liquid was validated using Western blot.

3.2.5.3 Measurement of concentration

In order to enable the measurement of Endo180 concentration, the solution needed to be concentrated to meet the measurement ranges of the equipment and the protein assay. The purified Endo180 protein solution was concentrated using EMD Millipore Amicon™ Ultra-0.5 Centrifugal Filter Units at 4°C. Two different methods, UV spectrophotometry (A_{280}) and bicinchoninic acid (BCA) assay, were used to measure the concentration of Endo180, and compared with each other to assure the accuracy of the result.

3.2.5.3.1 UV spectrophotometry (A_{280})

In order to determine the concentration of Endo180 through the measurement of the protein's absorbance at 280 nm (A_{280}), we first need to calculate the extinction coefficient (ε_{280}) of Endo180. The extinction coefficient of Endo180 was calculated by its amino acid composition using the following equation (Pace et al., 1995):

$$\varepsilon_{280} = (\text{no. of Trp}) \times 5500 + (\text{no. of Tyr}) \times 1490 + (\text{no. of Cys}) \times 125$$

(no. – number, Trp – Tryptophan, Tyr – Tyrosine, Cys – Cysteine)

The amino acid composition of Endo180 was obtained from NCBI (reference: NP_006030.2). After determining the extinction coefficient, the A_{280} absorbance of the

solution was measured using NanoDrop 2000 spectrophotometer, and the concentration of Endo180 was calculated using the following equation:

$$\textbf{Protein concentration (mg/ml)} = \frac{A_{280} \times molecular\ weight}{\varepsilon_{280} \times b}$$

(b – path length, cm)

3.2.5.3.2 BCA Assay

The concentration of verified Endo180 protein solution was also measured using BCA protein assay. Pierce™ BCA Protein Assay Kit was used to perform the assay. Diluted albumin standard set and BCA working reagent were prepared according to the manufacturer's manual. The assay was optimised as described below to minimise the consumption of Endo180 protein and guarantee the accuracy of the assay: 4 µL of each albumin standard and the Endo180 protein solution to be tested was added into a 0.2 mL tube with cap that contains 80 µL of BCA reagent and mixed thoroughly. All standard and samples were analysed in replicate. The tubes were closed and incubated at 37℃ for 3 hours. The tubes were cool to room temperature and applied to absorbance measurement and concentration calculation using NanoDrop 2000 spectrophotometer and the affiliated software.

3.2.6 Development and optimisation of Endo180 ELISA

3.2.6.1 Endo180 standard diluent

The Endo180 protein standard was diluted to 64 ng/mL, aliquot to 400 µL per tube and stored at -20℃. Prepare standard serial dilutions within 15 minutes before use. Take the stock solution out and allow it to sit for a minimum of 10 minutes with gentle agitation prior to dilution. Pipette 200 µL of standard diluent into each tube. Use the stock solution to produce a dilution series (Figure 3.1). Mix each tube thoroughly before

the next transfer. The stock solution serves as the highest standard. Standard diluent serves as the zero standard.

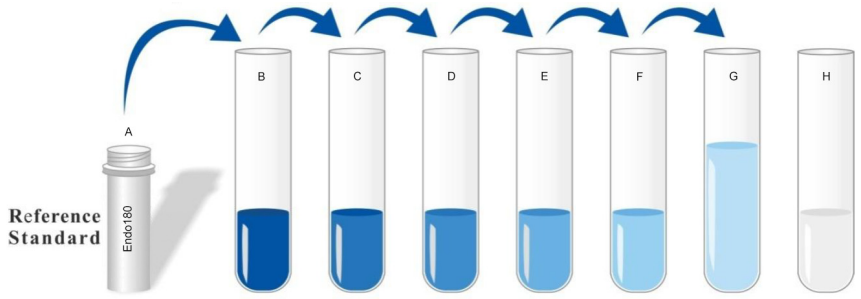


Figure 3.1 Preparation of standard dilution series
Take 200 µL of stock solution (tube A), add it to tube B with 200µL standard diluent and mix well to make standard solution in tube B. To prepare standard solution in tube C, take 200 µL solution from tube B and add to tube C with 200 µL standard diluent. Procedures to prepare the remained tubes (D-G) are all the same. The undiluted standard serves as the highest standard (tube A), and the Standard diluent serves as the zero standard (tube H).

3.2.6.2 Assay procedure

Bring all reagents and samples to room temperature before use. Centrifuge samples again after thawing before the assay. Add 100 µL coating antibody to each well of a 96-well microplate, cover with an adhesive strip and incubate overnight at 4°C on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. On the second day, aspirate and discard coating antibody and wash the plate by filling each well with wash buffer, shaking for 5 minutes, and removing wash buffer completely. Repeat the process twice for a total of three washes. After the last wash, remove any remaining wash buffer by inverting the plate and blot it against clean paper towels. Add blocking buffer per well, cover with a new adhesive strip and incubate for 1 hour at room temperature on the shaker. Aspirate the solution and wash each well three times as described above. Add standard/sample into each well, cover with a new adhesive strip and incubate for 1 hour at room temperature on the shaker. Aspirate the solution and wash each well three times as described above. Add detection antibody per well, cover with a new adhesive strip and incubate for 1 hour at room temperature on the shaker.

Aspirate the solution and wash each well three times as described above. Add enzyme conjugate working solution to each well, cover with a new adhesive strip and incubate for 1 hour at room temperature on the shaker. Aspirate the solution and repeat the wash process for six times. Add substrate solution to each well, cover with a new adhesive strip and incubate for ~15 minutes at room temperature on the benchtop, protect from light (the reaction time can be shortened or extended according to the actual colour change, but not more than 30 minutes). When apparent gradient appeared in standard wells, terminate the reaction by adding stop solution to each well, gently tap the plate to ensure thorough mixing. The colour in the wells should change from blue to yellow. The optical density of each well should be measured within 30 minutes, using a microplate reader set to 450 nm and 540 nm. Wavelength correction is applied by subtracting readings at 540 nm from the readings at 450 nm. A standard curve is created by plotting the average blank-corrected measurement for each standard versus its concentration using a five-parameter logistic regression algorithm. The concentration of each unknown sample was calculated by fitting into the standard curve.

3.2.7 Patient sample preparation

The design of this study was carried out under the guidance of Standards for Reporting of Diagnostic accuracy studies (Bossuyt et al., 2015). Ethical approval for the study was issued by the University of Liverpool (REC reference 09/H1010/54). The sample library has two cohorts: cohort 1 contains 26 plasma samples from patients who attended breast cancer services at Royal Liverpool University Hospital and were confirmed with breast cancer between 14 January 2014 and 18 March 2015; cohort 2 contains 59 plasma samples from Liverpool Bio-Innovation Hub Biobank. Patient samples were collected at diagnosis, and repeated collection was done for some of the patients 10 – 12 months later. Early (non-metastatic) breast cancer was defined as a

localised tumour that has not spread beyond the breast or the lymph nodes in the armpit on the same side of the body (Cancer Research UK, 2014b), which means the cancer has not spread to any other part of the body. Advanced (metastatic) breast cancer was defined as tumour that has spread to other parts of the body, mainly bones or visceral sites (Cancer Research UK, 2014b). When samples were collected, all early breast cancer patients were tested negative for recurrent disease, while metastatic breast cancer patients with bone metastasis showed evidence of osseous lesions determined by bone scintigraphy, computed tomography or magnetic resonance imaging, and patients with visceral metastasis showed lesions in visceral organs (lung or peritoneum) determined by radiological evaluation. Some patients had more than one metastatic sites.

Patient plasma collection and the measurement of conventional markers were carried out at Royal Liverpool University Hospital. For each sample, 10 mL of blood were collected from the patient and were transferred into a vacutainer blood collection tube with spray-coated K2EDTA. The tube was centrifuged (3,000 rpm) for 10 minutes at room temperature, and plasma was then aliquoted into screw cap microcentrifuge tubes. Samples were stored at -80°C freezer before use and frequent freeze thaw cycle were prevented in order to preserve the samples.

The concentrations of conventional bio-marker CA 15-3, BALP and CTX were measured by our collaborators at the University of Liverpool, and measurement results were transferred to the University of Hull together with patient information after patient samples were transferred and Endo180 measurement was done.

3.2.8 Western blot

Appropriate volume of protein-containing cell lysate (equal to 20 μg total protein) of each sample was mixed with 1/5 volume of sample buffer. Samples were then heated

to 65℃ for 5 minutes, cooled to room temperature and loaded on to 7.5% w/v discontinuous polyacrylamide gel (gels were prepared according to the formulation of Table 3.3). Electrophoresis was performed in 1× running buffer using the Mini-PROTEAN Cell System and the power pack set at 95V for about 1.5 hours until the bromophenol blue band was about to reach the bottom of the gel.

Table 3.4 Antibodies used for immunoblot analysis

Antigen (species)	*Clone (species)*	*Cat No.*	*Supplier*	*Stock conc. (mg/mL)*	*Dilution*	*Final Conc. (µg/mL)*
Endo180 (human)	A5/158 mAb (mouse)	N/A	self-produced	4	1:2000	2
β-actin (human)	mAb (mouse)	AM4302	ThermoFisher	2.9	1:2000	1.45
GAPDH (human)	mAb (mouse)	AM4300	ThermoFisher	2.9	1:2000	1.45
IgG (mouse)	pAb (rabbit)	A9044	Sigma	10	1:10000	1

Gels and membrane, filter paper and fibre pads were equilibrated and soaked in transfer buffer for 15 minutes. PVDF membranes were activated in methanol before being soaked in transfer buffer. Gel sandwich was assembled according to manufacturer's manual. The blot was run in transfer buffer at 100V, 4 ℃ for 1 hour. After the transfer finished, protein-containing membranes were blocked using blocking buffer for 1 hour at room temperature. Afterwards, each membrane was incubated with primary antibody solution in a centrifuge tube, which was placed on a universal turning device with continuous rotation at 4℃ overnight. After overnight incubation with the primary antibody, membranes were washed 3 × 10 minutes with washing buffer and incubated with secondary antibody for one hour at room temperature using the same method as the primary antibody incubation. After 3 × 10 minutes' washes, membranes were

incubated with ECL substrate for 3 minutes. Membrane exposure, image acquisition, and subsequent band analyses were carried out on ChemiDoc™ XRS+ System with associated ImageLab software (Bio-Rad).

3.2.9 Flow cytometry

Unless otherwise indicated, all reagents were precooled to 4 ℃ before use, and all the assay procedures were performed on ice to minimise the metabolism of the cell, so as to minimise the recycling of Endo180 between the cytoplasm and the cell membrane, in order to accurately measure the level of Endo180 on the cell surface under treatment conditions.

Cells were first blocked with blocking buffer for 20 minutes on ice. After the blocking, remaining blocking buffer was washed off using PBS. Primary antibody diluted in PBS were then added and incubated with the cells on ice for 20 minutes. Then the cells were washed using PBS for three times, and secondary antibody diluted in PBS were added and the tubes were put on ice for 20 minutes before being washed again with PBS for three times. 4% PFA solution were added to fix the cells, and then the cells were transferred into flow cytometry tubes and were ready for flow cytometry analysis.

Table 3.5 Antibodies used for flow cytometry

Antigen (species)	*Clone (species)*	*Cat No.*	*Supplier*	*Stock conc. (mg/mL)*	*Dilution*	*Final Conc. (µg/mL)*
Endo180 (human)	A5/158 mAb (mouse)	N/A	self-produced	4	1:400	10
Endo180 (human)	39.10 mAb (mouse)	N/A	self-produced	2	1:200	10
IgG (mouse)	pAb (rabbit)	A-11059	Invitrogen	2	1:200	10

Flow cytometry analysis was carried out using a FACSCalibur™ flow cytometer with associated BD CellQuest™ Pro data collection and analysis software. Samples installation, parameter optimisation, data acquirement and analysis were carried out under the instructions on manufacturer's manual.

3.2.10 Apoptosis assay

After treatment, cells were collected and washed with PBS. Cells were fixed using 1% w/v PFA (in PBS) on ice for 15 minutes, and then washed with PBS twice. Then the DNA break sites in cells were labelled and stained using the DNA-labeling solution provided in the APO-BrdU™ TUNEL Assay Kit with Alexa Fluor® 488 Anti-BrdU. Stained cells were analysed by flow cytometry, and the level of apoptosis was determined by comparing the geometric mean of the fluorescent intensity of the treatment groups with that of the positive and negative control group.

3.2.11 Gelatin zymography

Collected conditioned medium was mixed with 4× sample buffer and was then loaded in polyacrylamide gels containing 0.1% w/v gelatin. Electrophoresis was performed at 100V until the bromophenol blue tracking dye reaches the bottom of the gel. After electrophoresis, the gel was incubated with renaturing solution for 30 minutes. The buffer was then switched to developing buffer and incubated overnight at 37°C. The gel was stained with staining solution and then destained with destaining solution. The unstained bands reflect the areas of gelatin digestion. Bands were quantified using ChemiDoc™ XRS+ System.

3.2.12 Power calculation

In the pilot study that formed the basis of this study (Palmieri et al., 2013), one group of patients with metastatic disease who did not receive bisphosphonate treatment presented with an Endo180 mean ± standard deviation level of 2.17 ± 0.79 and the

group that received bisphosphonate treatment presented an Endo180 mean ± standard deviation level of 1.37 ± 0.74. These two groups have similar standard deviations and were therefore appropriate to use in a power calculation to determine how large the sample size would need to be to see a statistical difference in this study. Power calculation was carried out using the following two parameters, to determine the total number of patient needed and the number of patient needed in each group.

Parameter 1 (determine total patient number):

- Test chosen: two tailed t-test
- Significance level (adjusted for sidedness) = 0.025
- Standard deviation = 0.79
- Number of patients = undefined
- Power = 0.95
- Difference between means = 1.34

Parameter 2 (determine patient number needed in each group):

- Test chosen: sample size for unpaired t-test
- Significance level (α) = 0.05 (two-tailed)
- Expected standard deviation of each group = 0.79
- Number of patients = 12
- Power = 0.95
- Difference between means = 1.22

3.2.13 Statistical analysis

All results are expressed as mean ± standard error of mean (SEM). The distribution of the variables was tested by Kolmogorov–Smirnov test. Comparisons of the test variable between two independent groups were performed using Student's t-test, and nonparametric Mann-Whitney test was used for non-normal distributions where Student's t-test is not applicable. Comparisons of the test variable between multiple groups ($n \geq 3$) were carried out using one-way analysis of variance (ANOVA), and nonparametric Kruskal-Wallis test was used as a replacement where ANOVA is not applicable due to poor variance homogeneity. The potential relationships between

variables was analysed using linear regression analysis with one-way ANOVA or Spearman correlation test. In clinical sample analysis, receiver operating characteristics (ROC) curve analysis were used to evaluate the diagnostic accuracy of all tumour markers. The significant level was set at $P < 0.05$.

4 Development of the enzyme-linked immunosorbent assay for Endo180

4.1 Overview

Endo180, an endocytic receptor that interacts with extracellular and intracellular collagens, has been reported to be correlated with tumour grade, disease progression and poor prognosis in breast cancer (Wienke et al., 2007). Recent study showed that Endo180 ectodomain is released from breast cancer cells, and postulated that plasma Endo180 is a candidate tumour marker for metastatic breast cancer (Palmieri et al., 2013a).

In previous studies, the measurement of Endo180 in patient plasma and cell culture medium was mainly performed using Western blot. In this study, we have established a new ELISA system that detects Endo180 at a higher sensitivity and specificity, to facilitate further consideration of Endo180 in a broader clinical setting.

This chapter provides a detailed description of the development of Endo180 ELISA, including the preparation of prerequisite monoclonal antibodies, the production and quantification of standard protein, as well as the procedure setup and assay optimisation.

4.2 Experimental setup

As a premise for the clinical study, the Endo180 ELISA was designed and established, to accurately quantify Endo180 in patient plasma samples. Two hybridoma-produced mouse monoclonal antibodies that bind different epitopes on Endo180 ectodomain were used as the antibody-pair for Endo180 ELISA. The antibodies were purified by affinity chromatography using protein G column. Endo180 protein to be used as standard protein was produced by MCF7-E, a human breast cancer cell line that was transfected with pcDNA3-Endo180 vector to enable the expression of Endo180. Crosslink immunoprecipitation was used to isolate Endo180 from cells lysates. The

antibodies and standard protein were both verified and quantified before being used in ELISA development. Following the successful preparation of antibodies and standard protein, the development and optimisation of the Endo180 ELISA were carried out to determine the best parameters (Figure 4.1). Once optimised, the assay was ready for the clinical study to measure the level of Endo180 in patient samples, as well as to be used in other related studies.

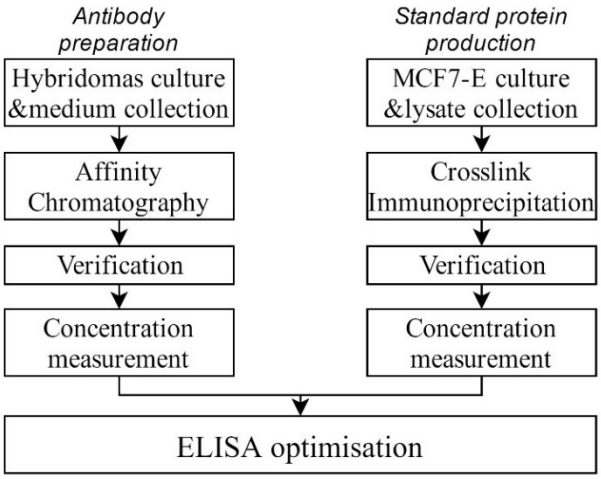


Figure 4.1 development of Endo180 ELISA
The two main preconditions for ELISA development were: 1) preparation of antibodies that perform well in the assay and 2) the production of standard protein that could precisely reflect the concentration of the unknown samples.

4.3 Results

4.3.1 Anti-Endo180 mAb A5/158 and 39.10 were produced and purified

After continuous culture and expansion of the two strains of hybridomas for eight weeks, 500 mL of antibody-containing medium from each strain was collected. The collected medium was then filtered and applied to HiTrap™ Protein G HP column, followed by desalting and buffer exchange. A total volume of 10 mL of each antibody were collected after purification. The purified antibodies were applied to SDS-PAGE, and the result validated the purity of both antibodies (Figure 4.2). The performance of both antibodies was also verified in the Western blot experiments carried out in Chapter 5 – 7.

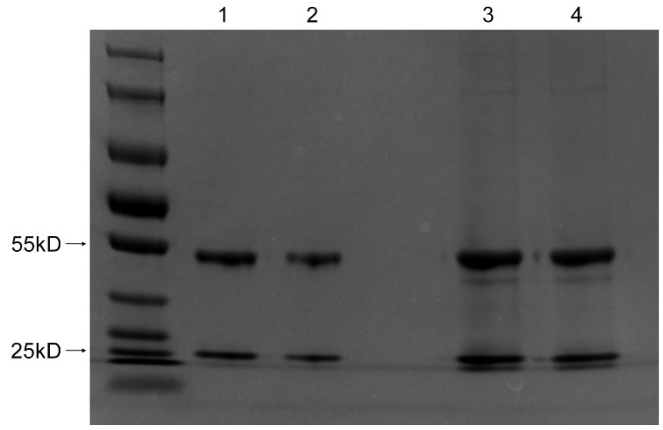


Figure 4.2 Verification of purified antibodies
Purified mAb A5/158 and 39.10 were verified in SDS-PAGE under reducing conditions to examine their purity. Heavy chains (55kDa) and light chains (25kDa) were separated during the electrophoresis. 1 & 2 – 39.10, 3 & 4 – A5/158.

4.3.2 Endo180 protein standard was extracted and quantified

In order to determine the concentration of Endo180 in unknown samples (patient plasma, cell culture media or cell lysate), purified Endo180 protein that has its concentration precisely measured is needed in the development of ELISA, to be used as standard protein. The availability of Endo180-overexpression cell lines and Endo180-binding antibodies in the laboratory made it possible to produce, purify, and measure the concentration of Endo180 protein for the development of Endo180 ELISA.

Full length Endo180 protein was extracted from MCF7-E cell lysate using crosslink immunoprecipitation. Eluent was applied to Western blot to check the effectiveness of the immunoprecipitation, as well as the rough amount of Endo180 protein collected (Figure 4.3). The result showed the extraction procedure went on well, and most of the Endo180 protein in the lysate successfully bond to the crosslinked antibody during the binding procedure of immunoprecipitation was eluted in the first wash. There was still detectable amount of Endo180 protein eluted in the second wash, but the amount was very small in comparison to the first wash. Endo180 could also be detected from the flow-through after the binding procedure, which indicates that the amount of Endo180

protein in the cell lysate that was added to the column was more than the binding capability of antibody.

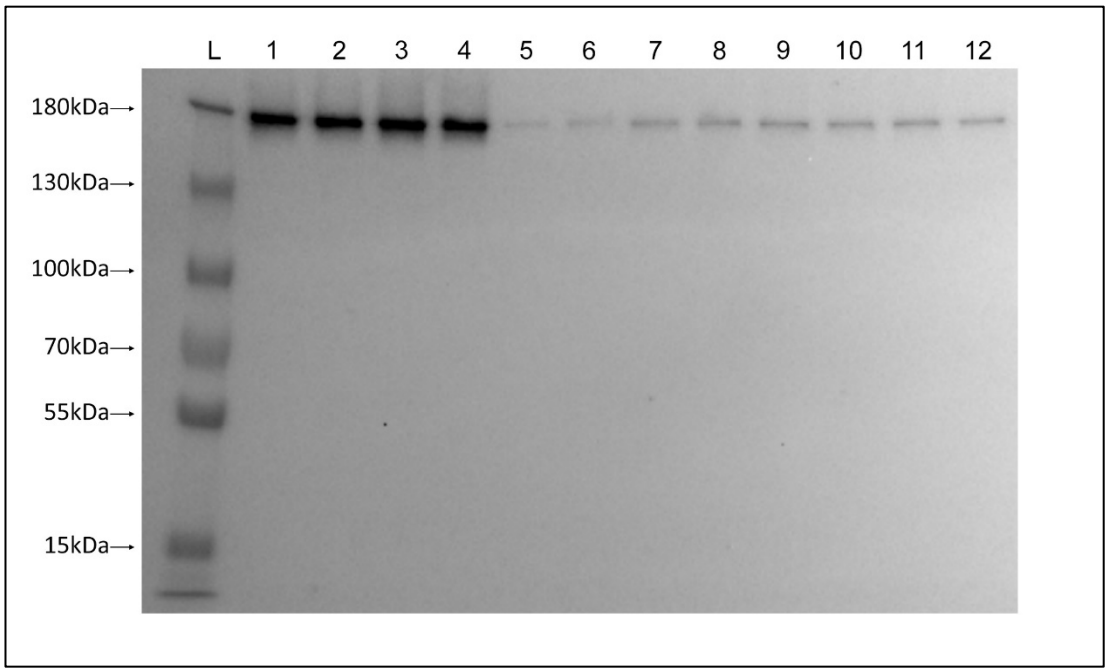


Figure 4.3 Verification of eluent from Endo180 immunoprecipitation
To confirm the successful binding and elution of Endo180 in immunoprecipitation, the eluent was tested in Western blot under non-reducing condition against anti-Endo180 mAb A5/158. Four independent repetitions were shown in this figure. Lane 1-4: eluent after first elution; Lane 5-8: eluent after second elution; Lane 9-12: spent cell lysate flow-through after the binding procedure.

After continuous immunoprecipitation experiments, we collected 20 tubes of 60 µL Endo180-containing eluates, which were validated in Western blot. These eluates were mixed into 2 tubes, resulting in 600 µL of eluates in each tube. The 2 tubes of eluates were then concentrated using centrifugal filter units separately, and 2 × 20 µL of concentrated Endo180 solutions were obtained. The two individually concentrated Endo180 solutions were then quantified using both UV spectrophotometry and BCA assay.

For UV spectrophotometry method, we first calculated the extinction coefficient (ε_{280}) of Endo180. Based on the sequence data obtained from NCBI, we found out the number of the three specific amino acids in Endo180 protein: Trp = 67, Tyr = 40, Cys = 56, and

determined the coefficient of Endo180: ε_{280} = 435,100 $M^{-1}cm^{-1}$. Then we measured the A_{280} of the two concentrated Endo180 solutions, and worked out the actual concentrations of Endo180 was 0.113 mg/mL and 0.106 mg/mL respectively. These results were highly consistent with the measurement results of the same samples in BCA assay, which indicates that the concentrations we obtained from the two assays were accurate, and the corresponding Endo180 solution could be diluted and used as standard protein in Endo180 ELISA.

4.3.3 Endo180 ELISA was established

4.3.3.1 Assay optimisation

Table 4.1 ELISA optimisation – example layout of checkerboard titration experiments

	0.5 µg/ml detection						1 µg/ml detection					
	1	2	3	4	5	6	7	8	9	10	11	12
	2 µg/ml capture	2 µg/ml capture	4 µg/ml capture	4 µg/ml capture	8 µg/ml capture	8 µg/ml capture	2 µg/ml capture	2 µg/ml capture	4 µg/ml capture	4 µg/ml capture	8 µg/ml capture	8 µg/ml capture
A	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
B	16 ng/ml standard	16	16	16	16	16	16 ng/ml standard	16	16	16	16	16
C	32 ng/ml standard	32	32	32	32	32	32 ng/ml standard	32	32	32	32	32
D	64 ng/ml standard	64	64	64	64	64	64 ng/ml standard	64	64	64	64	64
E	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
F	16 ng/ml standard	16	16	16	16	16	16 ng/ml standard	16	16	16	16	16
G	32 ng/ml standard	32	32	32	32	32	32 ng/ml standard	32	32	32	32	32
H	64 ng/ml standard	64	64	64	64	64	64 ng/ml standard	64	64	64	64	64
	2 µg/ml detection						4 µg/ml detection					

To start the optimisation of the assay, an initial setup of the Endo180 ELISA was determined using the upper limits of the recommended concentrations for all reagents by general ELISA development protocols, which includes: the coating antibody at 16 µg/mL, the detection antibody at 8 µg/mL, and the HRP-conjugated enzyme at 200 ng/mL. Serial dilutions of Endo180 standard protein within a concentration range between 1 ng/mL and 500 ng/mL were used to test the sensitivity and capability of the assay. High background signals were detected in assays using the initial setting, which

was considered to be a result of the extremely high concentrations of both coating and detection antibodies used in the initial assay, or a result of the concentrations of the standard protein higher than assay capability.

After standard curve was obtained using the initial setup, optimisation of all parameters was carried out in order to improve the performance of the assay. There are many different factors that could influence the reliability and sensitivity of ELISA, which includes the concentrations of coating and detection antibody, incubation time and temperature, the type and concentrations of enzyme conjugate and substrate. Among these components, the concentrations of capture antibody and detection antibody are the major two factors that would determine the accuracy and sensitivity of the assay. A series of checkerboard titration experiments were performed to optimise these two factors simultaneously.

In the checkerboard titration experiment, a 96-well microplate was divided into 4 sections. The six columns in each section represented three different concentrations of the capture antibody, the four rows in each section represented three different standard concentrations, and the four sections represented four different concentration of detection antibody. For example, in the layout as shown in Table 4.1, each section contained all the possible combinations of capture antibody concentrations of 2, 4 and 8 μg/mL and standard concentrations of 0, 16, 32, and 64 ng/mL, at one detection antibody concentration (0.5, 1, 2, or 4 μg/ml). The 0 ng/mL standard concentration gives the background value that could be expected at each of the antibody pair concentrations and the 16, 32, and 64 ng/mL standard concentrations gave the signal resulting from each of the antibody concentration pairs. The concentration pair of capture antibody and detection antibody that result in the best signal to background ratio (≥ 5) was chosen.

4.3.3.2 Antibody pair

After the optimisation, the new ELISA system using anti-Endo180 mAb A5/158 and 39.10 was established and evaluated by measuring the serial dilutions of the quantified Endo180 standard protein. The two antibodies could be used in either order, A5/158 as capture antibody with 39.10 as detection antibody, or 39.10 as capture antibody with A5/158 as capture antibody. The performances of both combinations have been compared and no significant difference was detected (Figure 4.4).

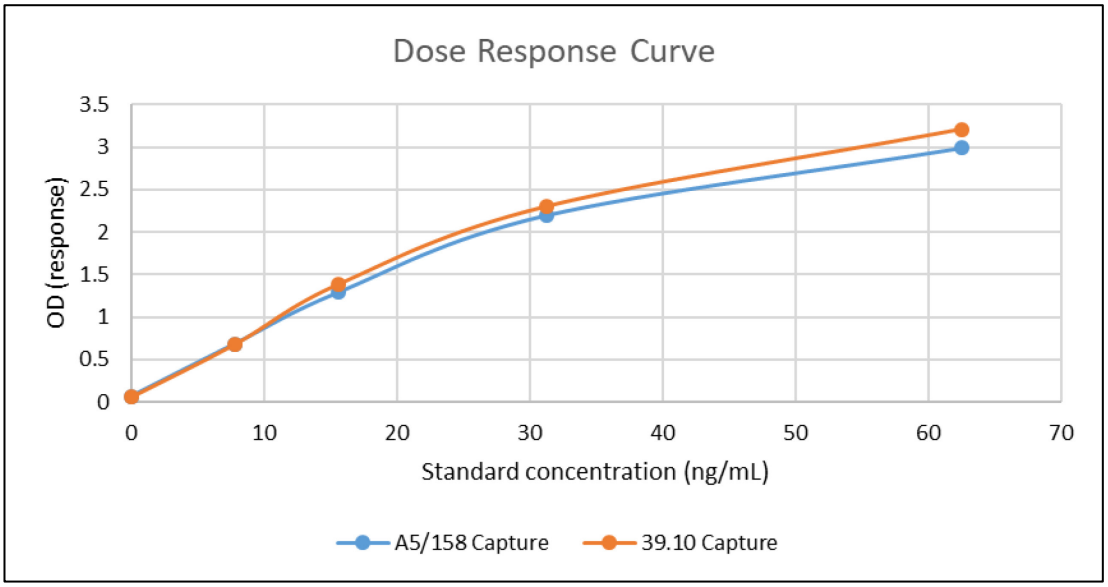


Figure 4.4 Comparison of antibody pairs

The two anti-Endo180 mAbs, A5/158 and 39.10, were used as capture antibody and detection antibody respectively. Two combinations of the antibody pair: 1) A5/158 as capture antibody with 39.10 as detection antibody, and 2) 39.10 as capture antibody with A5/158 as capture antibody, were both carried and analysed during the assay optimisation. The results indicate that under the same concentrations of the capture antibody and detection antibody, both antibodies could be used as either the coating antibody or the detection antibody, and there was no significant difference in performance between the two combinations.

4.3.3.3 Comparison between conventional sandwich ELISA and the "ABC" system

Based on the sandwich type ELISA, we also compared the two approaches to generate the chemical the using avidin-biotin binding: 1) the conventional method uses a biotinylated detection antibody, which is probed using streptavidin protein conjugated to horseradish peroxidase (HRP) enzymes; 2) the "avidin-biotin complex" (ABC)

system, which also employs a biotinylated detection antibody, but the antibody is probed with a pre-incubated mixture of avidin and biotinylated enzyme. Both methods could generate amplified signal, because biotinylating procedure usually adds multiple biotin tags to one antibody molecule, thus allowing more than one streptavidin molecule to bind to each antibody (Figure 4.5).

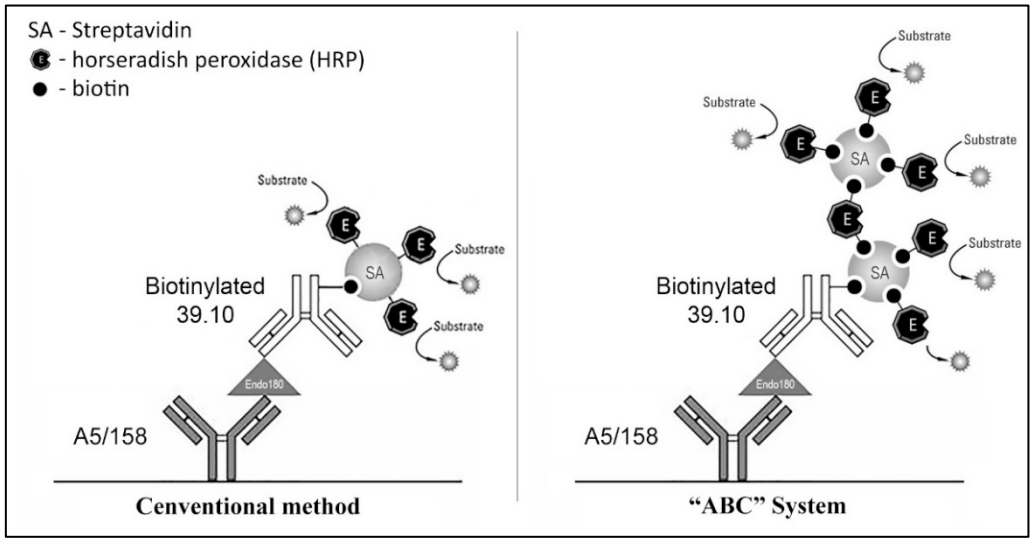


Figure 4.5 The difference between conventional sandwich ELISA and the "ABC" system Adapted from ELISA technical guide and protocols (Thermo Fisher Scientific, 2010)
The "ABC" system is considered to have higher signal amplification capability, as the use of a pre-incubated mixture of streptavidin plus biotinylated enzyme results in conjugates having more than one enzyme, thus increases the number of enzyme molecules in the final immune complex. This increases the catalysis of appropriate substrate and gives a stronger signal

In this study, after successfully developed and optimised sandwich ELISA using the conventional signal amplifying method, we also tried the "ABC" system, and compared its performance with the conventional method. Our result shows that there was no significant difference between the two methods, and the conventional method seems to have better higher maximum binding capability when using the same parameters (Figure 4.6).

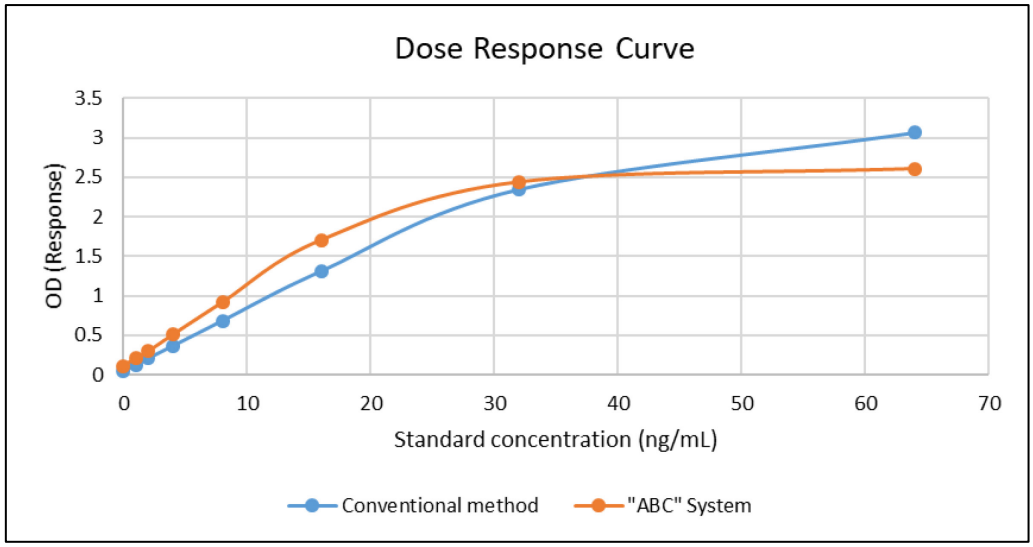


Figure 4.6 Performance of conventional method and "ABC" system

4.3.3.4 Established Endo180

The concentration pair of 8 µg/mL for coating antibody A8/158, and 2 µg/mL for detection antibody 39.10 was finally determined to have the best performance. After optimisation, the standard dose response curve exhibited a linear shape plotted over a range of 1.56 – 100 ng/mL calculated with Endo180 expressed in MCF7-E cells transfect with pcDNA3-Endo180 vector as standard protein (Figure 4.7).

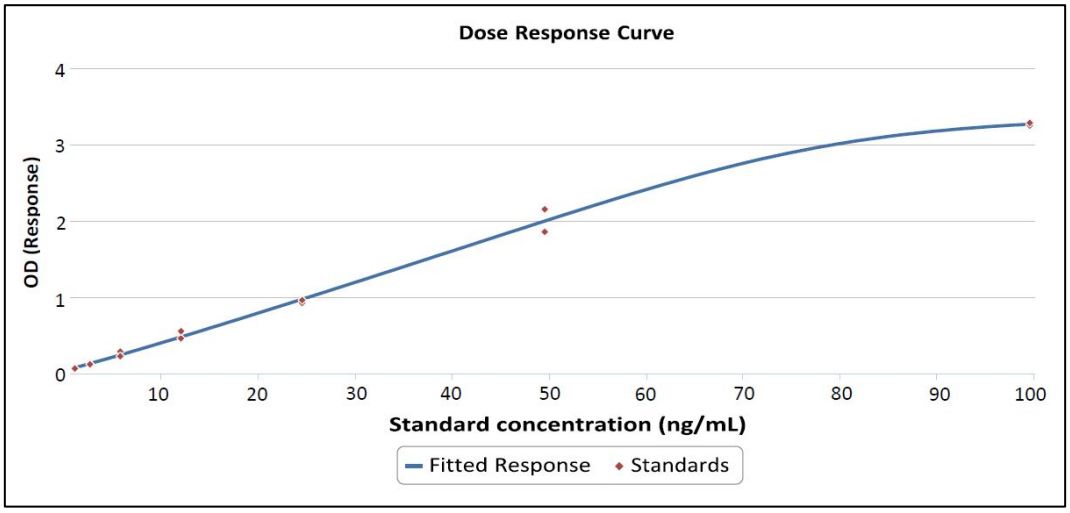


Figure 4.7 Standard protein dose response curve for Endo180 ELISA

The standard curve was generated by plotting the average optical density (OD) readings of each standard (wavelength-corrected) versus the concentration, using the five-parameter logistic regression algorithm. $R^2 > 0.99$.

4.4 Discussion

ELISA is an ideal method to measure clinical and research samples, as it has the advantage of detecting the target protein sensitively, quantifying protein concentration accurately, and analysing mickle samples simultaneously. The successful development of Endo180 ELISA in this study brings improvements in accuracy and throughput in comparison to conventional immunoassays used to detect Endo180 in previous studies.

The Endo180 ELISA developed in this project is designed to use a Sandwich ELISA assay type (Figure 4.8). This design is determined by a comprehensive consideration of the advantages / disadvantages of each assay type, as well as the reagents available. First, the main advantage of Sandwich ELISA in comparison to other assay types is its high specificity, which is very important in clinical application. Second, the sandwich style could increase the sensitivity of the assay, which is essential to the research application of the assay, as both the level of Endo180 in patient plasma and the level released by cells *in vitro* is relatively low. Third, there are two monoclonal antibodies that detect two different epitopes on Endo180, which makes it possible to design the sandwich ELISA. Considering all above conditions, an indirect sandwich design is the best option for the development of ELISA to measure Endo180.

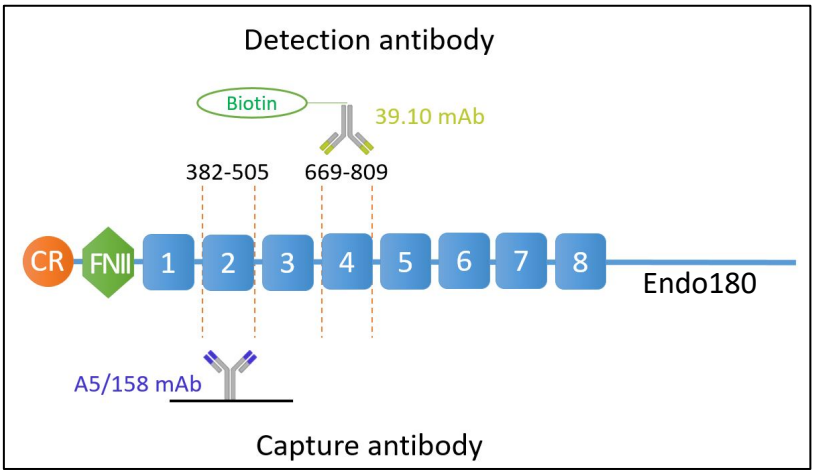


Figure 4.8 The design of Endo180 ELISA
Endo180 ELISA is designed to be an indirect Sandwich ELISA, in which mAb A5/158 was used as the coating antibody and biotinylated 39.10 acted as detection antibody.

There are several obstacles that block the progression during the development. The first and fundamental part of the development of an ELISA is to find a good antibody pair that can perform well with each other. The two anti-Endo180 monoclonal antibodies available in our lab, A5/158 and 39.10, which recognise different epitopes on Endo180 (Figure 2.3), meet this requirement. The recovery of cryopreserved hybridomas saved the time of generating fresh antibody-expressing cell lines and provide adequate antibodies for the development. With the help of commercially available columns pre-bound with protein G, the two antibodies were successfully extracted and purified.

The second obstacle was to produce enough Endo180 protein and precisely quantify it as standard protein. In order to carry out quantitative analysis using ELISA, a dilution serious of precisely measured standard protein is required to be added alongside blank and unknown samples in each experiment. Instead of trying to express the protein using yeast or prokaryotes such as *E. coli*, expression and extraction of Endo180 under natural state from a mammalian cell line could guarantee the tertiary structure and antibody binding capability of the protein. The only problem was the naturally low expression levels of Endo180 in common immortal mammalian cell lines. This is solved by the use of MCF7-E cells, which is transfected with Endo180 encoding vector to overexpress the protein as described previously (Wienke et al., 2003).

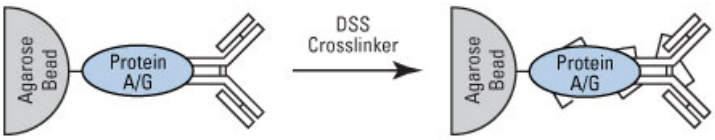


Figure 4.9 The mechanism of crosslink immunoprecipitation
After binding of antibody to protein A/G plus agarose, DSS crosslinker was used to crosslink the bound antibody with protein A/G, which increases the binding so that the crosslinked antibody can hardly be eluted together with antigen during the elution steps. Reproduced from *Immunoprecipitation (IP) technical guide and protocols* (Thermo Fisher Scientific, 2009)

A special crosslink immunoprecipitation method was used to isolate Endo180 protein from cell lysate. In comparison to conventional immunoprecipitation, the crosslink method combines the high-binding capacity of protein A/G agarose with the reliable crosslinking chemical property of DSS, which covalently crosslinks antibodies onto the resin, so as to ensure high antigen yield with minimum antibody co-elution during the elution steps (Figure 4.9).

Endo180 protein was collected using crosslink immunoprecipitation, but the relatively low concentration of the eluted protein solution could not meet the limited measuring range of the commonly used methods, such as UV spectrometry and BCA assay. Therefore, a scale up of the immunoprecipitation was performed and repeated. The concentrations of the concentrated solutions were measured with two methods, UV spectrometry and BCA assay, to ensure the accuracy. Because we had limited amount of the concentrated solutions, and the traditional BCA assay requires a large amount of protein sample which cannot be reused after the quantification, we optimised the assay to minimise sample consumption and improve the sensitivity. The concentrated sample was successfully quantified and the results of the two assays were highly consistent. The standard protein was diluted and the aliquots were stored at -20℃ for future use.

The third obstacle was to optimise the assay to meet the performance requirement. There are several factors which could affect the accuracy and the sensitivity of ELISA, which include the concentrations of capture antibody and detection antibody, dilution level of standard protein and unknown samples, the concentration of enzyme conjugate, incubation temperature, incubation time, and washing efficiency. Each component has its own influence on the assay, and they also rely on or act together with other components to determine the performance of the assay. Finding the best combination of all components is the precondition of a good assay. An incorrect parameter could

influence the whole assay, and is hard to be found out due to the large number of different parameters. An initial assay was carried out using the highest recommended reagent concentrations and the suggested procedures to verify that all suggested procedures works, although no perfect results were obtained. As the concentrations of the antibody pair are considered to be the most important factor that decides the performance, checkerboard titration experiments were carried out to find out the best antibody concentration pair that result in a high signal to noise ratio. Once the best concentration pair is determined, experiments using a wider dilution range of standard protein were performed to check the measuring range of the assay. Some other parameters such as incubation time and washing procedures were also adjusted at this stage. The optimised parameters were also repeated to verify their stability.

Before the assay was ready to apply to clinical and biological studies, it is necessary to test the assay using serum containing samples, to ensure that it could not only detect the purified Endo180 in PBS, but also accurately measure the level of Endo180 in more complicated samples, such as patient blood and cell culture medium. A spiked standard protein solution with human or bovine serum as the solvent would be able to test the capability of the assay, as well as the detection range in a complicated environment.

5 The investigation of Endo180 as a biomarker for advanced breast cancer

5.1 Overview

As described in Chapter 2, tumour markers are useful in the detection, diagnoses, and/or management of cancer progression. A tumour marker for breast cancer, which could accurately detect the occurrence of metastases and predict the prognosis of patients, is an unmet clinical need. It is suggested that the dissemination of tumour cells to the secondary sites happens at the early stages of primary cancer, while the growth of secondary tumours to a detectable size occurs much later, sometimes many years after the first diagnoses of the disease (Riethmüller and Klein, 2001). This also highlights the importance of tumour markers that could indicate the presence of metastases at early stage of cancer progression.

Previous research showed that Endo180 level is increased in the plasma of breast cancer patients with bone or visceral metastasis, and measurement of plasma Endo180 level alone, or in combination with CA15-3, has a higher diagnostic accuracy for the classification of metastasis (Palmieri et al., 2013a). From these findings, we hypothesised that the level of plasma Endo180 is increased in patients with advanced breast cancer, and could be used as a potential biomarker for advanced breast cancer.

The aim of this study was to investigate the correlation of plasma Endo180 level with the pathological characteristics of breast tumours, the difference of plasma Endo180 levels between early and advanced breast cancer patients, and compare Endo180 with other breast cancer biomarkers.

From the preliminary data collected in this study, we found that plasma Endo180 levels in advanced breast cancer patients with bone/visceral metastases were significantly higher than the level of plasma Endo180 in early breast cancer patients, which indicates

that plasma Endo180 levels in breast cancer patients could be used as an indicator of advanced breast cancer.

5.2 Experimental setup

In order to verify plasma Endo180 as a potential biomarker for advanced breast cancer, statistical analyses were carried out to compare the level of Endo180 in the plasma of breast cancer patients at different disease stages (early and advanced). Analyses includes: a) confirm potential influences of patients' pathological characteristics (e.g. age, hormone receptor, treatment) on plasma Endo180 level; b) compare plasma Endo180 levels between early and advanced breast cancer patients, c) work out the receiver operating characteristic (ROC) of plasma Endo180 in advanced breast cancer prediction and determine the threshold value, d) compare Endo180 with other biomarkers that are commonly used in breast cancer diagnoses and research (Figure 5.1).

Based on the design above, it was determined that the samples used in this study was the plasma collected from early and advanced breast cancer patients. Information of the patients required for analysis include date of birth, current status (alive or dead), date of death, stage of disease, date of sample collection, hormonal receptor status, date and type of treatments received (bone therapy and other therapies). The patient samples were collected in cooperation with Professor Carlo Palmieri's research group at the University of Liverpool, where the conventional biomarkers (CA 15-3, BALP and CTX) were also measured upon collection (for some reason, the serial collection of patient plasma designed for aim II was not implemented, and the measurement of conventional markers was only done with some of the samples). The required information of patients (without personal identification information), together with the measurement results of the conventional biomarkers, was transferred together with samples to the University

of Hull, where Endo180 quantification and further analyses were implemented (Figure 5.1). For Endo180 analysis, The Endo180 ELISA developed as described in Chapter 4 was used.

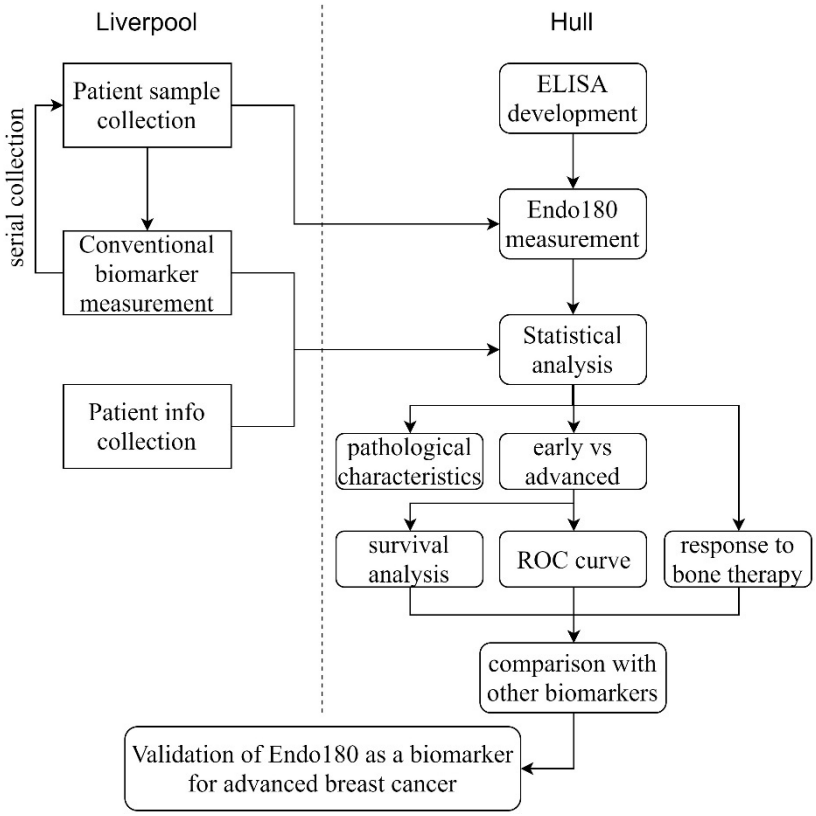


Figure 5.1 Design of the clinical study to investigate Endo180 as a potential biomarker for advanced breast cancer

Originally, this study was also designed to investigate the dynamical changes of plasma Endo180 levels throughout bone therapy (bisphosphonates or denosumab treatment) in breast cancer patients with bone metastasis (Figure 5.1). The plasma Endo180 levels in breast cancer patients with bone metastasis were supposed to be regularly monitored (3, 6, and 12 weeks) from the outset of their bone therapy, and the trend of plasma Endo180 levels throughout bone therapy would be analysed, together with the patient's response to the therapy. However, when the study actually started, no related sample or patient information was provided by our collaborator.

5.3 Patient information

5.3.1 Cohort 1

This section contains the information of cohort 1 breast cancer patients, from whom the plasma samples were collected by our collaborators at the University of Liverpool between 2014 and 2015. The samples were then transferred to the University of Hull for Endo180 measurement. According to the information released by the sample provider after experiments were done, there were 26 samples from 23 patients (with 3 patients who had repeated plasma collections). However, 3 samples which were on the list were not provided for experimental analysis due to unknown reason.

Table 5.1 Basic information of Cohort 1 breast cancer patients

No.	*DoB*	*DoC-1*	*DoC-2*	*Current status (date)*	*Stage*
1	26/01/1951	19/02/2014		RIP (09/07/2015)	Advanced
2	14/10/1946	26/02/2014		RIP (09/07/2015)	Advanced
3	22/11/1950	26/02/2014		Alive	Advanced
4	16/11/1941	05/03/2014	14/01/2015	RIP (16/02/2016)	Advanced
5	06/06/1943	12/03/2014	14/01/2015	Alive	Advanced
6	06/08/1950	19/03/2014	*16/04/2014	RIP (19/12/2015)	Advanced
7	07/03/1948	02/04/2014		RIP (02/03/2016)	Advanced
8	04/06/1954	14/01/2015		Alive	Early
9	24/08/1963	14/01/2015		Alive	Early
10	10/04/1940	21/01/2015		Alive	Advanced
11	07/06/1948	21/01/2015		Unknown	Early
**12*	30/09/1966	21/01/2015		Alive	Advanced
13	12/03/1938	04/02/2015		Alive	Advanced
14	28/04/1960	04/02/2015		Alive	Early
**15*	22/06/1936	04/02/2015		RIP (09/06/2015)	Early
16	14/05/1950	18/02/2015		Alive	Early
17	30/04/1957	18/02/2015		Alive	Early
18	16/09/1967	18/02/2015		Alive	Early
19	30/01/1956	18/02/2015		Alive	Early
20	30/11/1974	25/02/2015		Alive	Early
21	07/05/1937	18/03/2015		Alive	Early
22	13/01/1955	18/03/2015		Alive	Early
23	03/01/1967	18/03/2015		Alive	Early

Note:
*DoB – date of birth, DoC – date of sample collection, RIP – requiescat in pace, * – no sample*

Table 5.2 Hormonal receptor and hormonal therapy status

No.	*ER*	*PR*	*HER2*	*Hormonal therapy*
1	Positive	Negative	Unknown	Y
2	Positive	Negative	Unknown	Y
3	Positive	Unknown	Negative	Y
4	Positive	Negative	Unknown	Y
5	Positive	Positive	Negative	N
6	Positive	Positive	Unknown	Y
7	Unknown	Unknown	Unknown	Y
8	Positive	Unknown	Negative	Y
9	Positive	Unknown	Negative	Y (12/2013)
10	Positive	Unknown	Negative	Y (05/2013)
11	Negative	Negative	Negative	N
**12*	Negative	Negative	Negative	Y
13	Positive	Positive	Negative	Y (12/2013)
14	Positive	Unknown	Negative	Y
**15*	Negative	Negative	Negative	N
16	Negative	Negative	Negative	N
17	Positive	Unknown	Negative	**Y (03/2015)
18	Positive	Positive	Positive	**Y (04/2015)
19	Positive	Unknown	Negative	N
20	Positive	Unknown	Negative	Y
21	Unknown	Unknown	Positive	Y (09/2014)
22	Positive	Positive	Negative	Y
23	Unknown	Unknown	Negative	Y

Note:
*ER – estrogen receptor, PR – progesterone receptor, HER2 – human epidermal growth factor receptor 2; Y (date) – therapy received (receive date), N – therapy not received, ** – treatment date later than sample collection date, considered as no treatment*

Table 5.3 Metastatic site(s) of advanced breast cancer patients

No.	*Stage*	*Bone*	*Lung*	*Peritoneal*
1	Advanced	Y	Y	N
2	Advanced	N	Y	N
3	Advanced	Y	N	Y
4	Advanced	Y	N	N
5	Advanced	Y	N	N
6	Advanced	Y	Y	N
7	Advanced	Y	N	N
10	Advanced	Y	N	N
**12*	Advanced	N	Y	N
13	Advanced	Y	N	N

Note:
Y –metastasis found at this site, N – no metastasis found at this site

Table 5.4 Chemotherapy and bone therapy status

No.	*Chemotherapy*	*Bisphosphonate*	*Denosumab*
1	Y	Unknow	Y (01/2012)
2	Y (01/2005)	Unknow	Y
3	**Y (02/2016)	Unknow	Y (02/2014)
4	Y (01/2014)	Unknow	Unknow
5	Y (12/2003)	Unknow	Y (10/2013)
6	Y (03/2014)	ZA (11/2011)	Y (10/2014)
7	Unknow	Unknow	Y (11/2013)
8	Y (03/2014)	ZA (2014)	Unknow
9	Y (12/2013)	Unknow	Unknow
10	N	Unknow	Y (05/2013)
11	Unknow	Unknow	Unknow
**12*	Y (01/2014)	Unknow	Unknow
13	Unknow	Unknow	Y (07/2014)
14	Y (09/2014)	Unknow	Unknow
**15*	No	No	No
16	**Y (12/2015)	Unknow	Unknow
17	Y (10/2014)	Unknow	Unknow
18	Y (09/2014)	Unknow	Unknow
19	**Y (05/2015)	Unknow	Unknow
20	Y (04/2014)	ZA	Unknow
21	Y (05/2014)	Unknow	Unknow
22	Y	Unknow	Unknow
23	Y (10/2014)	Unknow	Unknow

Note:
*Y (date) – treatment received (date), N – treatment not received, ZA – zoledronic acid, ** – treatment date later than sample collection date, considered as no treatment*

Table 5.5 Measurements of conventional biomarkers

No.	*CA15-3*		*BALP*		*CTX*	
	conc. (U/mL)	date	conc. (U/L)	date	conc. (ng/mL)	date
1	20	24/02/2014	67	24/02/2014	0.07	24/02/2014
2	596	26/02/2014	240	26/02/2014	0.15	26/02/2014
3	100	26/02/2014	66	25/02/2014	0.73	26/02/2014
4	9	11/03/2014	62	05/03/2014	0.03	11/03/2014
5	/	/	46	12/03/2014	/	/
6	2100	19/03/2014	327	19/03/2014	/	/
7	34	02/04/2014	48	02/04/2014	0.11	02/04/2014
8	/	/	/	/	/	/
9	/	/	/	/	/	/
10	28	18/03/2015	89	20/01/2015	/	/
11	/	/	/	/	/	/
**12*	23	21/01/2015	104	21/01/2015	/	/
13	/	/	54	03/02/2015	/	/

14	/	/	/	/	/	/
**15*	/	/	/	/	/	/
16	/	/	91	17/02/2015	/	/
17	/	/		/	/	/
18	/	/	69	18/02/2015	/	/
19	/	/	59	18/02/2015	/	/
20	/	/	87	23/02/2015	/	/
21	/	/	/	/	/	/
22	/	/	/	/	/	/
23	/	/	90	11/03/2015	/	/
R4	/	/	82	13/01/2015	/	/
R5	/	/	46	06/01/2015	/	/

Note:

1. Measurement were done by collaborators at the University of Liverpool.

2. "/" – not done, "R4" – second sample from patient #4.

5.3.2 Cohort 2

As a supplement, the cohort 2 samples were collected at the University of Liverpool, and was transferred to the University of Hull for Endo180 analysis on January 2017. It contains 59 samples, among which 9 were samples from advanced breast cancer patients, and 50 from early breast cancer patients. The information revealed following the experiments were different between advanced and early stage patients, so they were divided into two groups and were described respectively.

(1) Advanced breast cancer patients

Table 5.6 Basic information of Cohort 2 breast cancer patients

#	*Hormonal receptor*			*Metastatic site(s)*				*Current status*
	ER	*PR*	*HER2*	*Bone*	*Peritoneum*	*Liver*	*Brain*	
1	+	?	-	+	+	-	-	Alive
2	-	+	-	-	-	+	-	Alive
3	+	?	-	-	-	+	-	Alive
4	+	+	+	+	-	-	-	Alive
5	-	-	-	+	-	+	-	RIP (10/05/2016)
6	+	?	-	+	-	-	-	Alive
7	-	-	-	-	-	+	-	RIP (21/06/2016)
8	+	+	?	+	-	+	+	RIP
9	+	+	-	-	-	+	-	RIP (08/08/2016)

Note:

"+" – positive, "-" – Negative, "?" – unknown

Table 5.7 Treatment status

No.	*Chemotherapy*	*Hormonal therapy*	*Bisphosphonates*	*Denosumab*
1	Unknown	Unknown	N	Y
2	N	Y	N	N
3	Y	N	N	N
4	Y	Y	Y	N
5	Y	N	N	Y
6	N	Y	N	Y
7	N	N	N	N
8	Unknown	Unknown	N	N
9	Y	N	N	N

Table 5.8 Measurements of conventional biomarkers

No.	*BALP (U/L)*	*CA15-3 (U/mL)*
1	80	/
2	90	/
3	306	/
4	105	/
5	95	24
6	135	273
7	997	666
8	/	/
9	727	>3000

Note:

1. Measurement were implemented by collaborators at the University of Liverpool.

2. "/" – not done.

(2) Early breast cancer patients

Table 5.9 TNM staging and hormonal receptor status of early breast cancer paitents

No.	*TNM staging*			*Hormonal receptor*		
	pT	*pN*	*pM*	*ER*	*PR*	*HER2*
10	1c	0	X	Positive	Unknown	Negative
11	1c	1mi		Positive	Unknown	Negative
12	1c	1mi		Positive	Unknown	Negative
13	1a	X	1c	Unknown	Unknown	Unknown
14	1c	0		Positive	Unknown	Negative
15	1c	0		Negative	Negative	Positive
16	1b	0		Positive	Unknown	Negative
17	1c	0		Positive	Unknown	Negative
18	1b	0		Positive	Unknown	Negative
19	1b			Positive	Unknown	Unknown
20	1b		1	Positive	Unknown	Negative
21	1c	0		Positive	Unknown	Negative
22	1			Positive	Unknown	Unknown

23	1c	0		Positive	Unknown	Positive
24	1c	1mi		Positive	Unknown	Negative
25	1c	1a		Negative	Negative	Negative
26	1a	1mi	X	Positive	Unknown	Negative
27	1c	0		Positive	Unknown	Negative
28	1c	0		Positive	Unknown	Negative
29	1b	0		Positive	Unknown	Negative
30	1a	0		Positive	Unknown	Negative
31	1	0		Negative	Negative	Unknown
32	1c	0		Positive	Unknown	Positive
33	1c	1mi		Positive	Positive	Negative
34	1c	1mi		Positive	Unknown	Negative
35	1c	0		Negative	Positive	Negative
36	1b	0		Positive	Unknown	Negative
37	1c	0		Negative	Negative	Positive
38	1b	0		Positive	Unknown	Negative
39	1c	1mi		Positive	Unknown	Negative
40	1c	0		Positive	Unknown	Negative
41	1c	0		Negative	Positive	Positive
42	1c	1mi		Positive	Unknown	Negative
43	1c			Positive	Unknown	Negative
44	1c	0		Positive	Unknown	Negative
45	1c	0		Positive	Unknown	Negative
46	1	2	1	Positive	Unknown	Negative
47	1c	X		Positive	Unknown	Negative
48	1c	0		Negative	Positive	Negative
49	1c	0		Negative	Negative	Positive
50	1b			Positive	Unknown	Negative
51	1c	0		Positive	Unknown	Positive
52	1b			Positive	Unknown	Borderline
53	1c	1a		Positive	Unknown	Negative
54	1c	0		Positive	Unknown	Borderline
55	1b	0		Positive	Unknown	Borderline
56	1c	0		Positive	Unknown	Borderline
57	1c	1a		Negative	Unknown	Negative
58	1c	1a	X	Negative	Negative	Positive
59	1c	1		Positive	Unknown	Negative

5.4 Results

5.4.1 Endo180 quantification

Table 5.10 Plasma Endo180 levels in patient samples

Cohort 1

#	Stage	Concentration (ng/ml)	#	Stage	Concentration (ng/ml)
1	A	40.254	*14*	E	55.508
2	A	60.348	*15*	E	Sample not provided
3	A	42.318	*16*	E	24.776
4	A	31.962	*17*	E	12.370
5	A	29.762	*18*	E	23.442
6	A	42.260	*19*	E	33.166
7	A	47.576	*20*	E	36.368
8	E	22.814	*21*	E	27.784
9	E	39.856	*22*	E	31.112
10	A	42.592	*23*	E	22.580
11	E	32.946	*R4*	A	22.312
12	A	Sample not provided	*R5*	A	25.050
13	A	30.262	*R6*	A	Sample not provided

Cohort 2

#	Stage	Conc. (ng/ml)	#	Stage	Conc. (ng/ml)	#	Stage	Conc. (ng/ml)
1	A	67.704	*21*	E	28.440	*41*	E	38.392
2	A	41.830	*22*	E	31.600	*42*	E	26.126
3	A	42.454	*23*	E	15.614	*43*	E	30.946
4	A	43.720	*24*	E	30.786	*44*	E	26.810
5	A	66.530	*25*	E	21.558	*45*	E	99.486
6	A	59.582	*26*	E	43.858	*46*	E	35.096
7	A	109.812	*27*	E	35.334	*47*	E	46.806
8	A	27.602	*28*	E	24.218	*48*	E	37.930
9	A	50.856	*29*	E	24.192	*49*	E	16.158
10	E	41.064	*30*	E	20.070	*50*	E	45.334
11	E	56.314	*31*	E	28.760	*51*	E	26.090
12	E	27.926	*32*	E	15.438	*52*	E	44.370
13	E	28.302	*33*	E	93.488	*53*	E	19.862
14	E	43.506	*34*	E	31.272	*54*	E	25.974
15	E	33.150	*35*	E	32.508	*55*	E	21.162
16	E	22.946	*36*	E	18.462	*56*	E	36.160
17	E	21.680	*37*	E	27.388	*57*	E	30.490
18	E	26.626	*38*	E	21.218	*58*	E	27.496
19	E	31.638	*39*	E	85.986	*59*	E	50.844
20	E	36.662	*40*	E	16.300			

Note: A – advanced, E – early.

5.4.2 Plasma Endo180 levels are not correlated with patients' pathological characteristics

Power analysis based on data from the pivotal study that forms the basis of this project showed that a sample size of 22 patients in total (or 12 patients in each group) has 95% power to detect a difference in means of 1.22 with a significance level of α=0.05 (two-tailed) between variables (50% confidence interval; 0.79 standard deviation).

The pathological characteristics of the patients in cohort 1 and the treatment they received were recorded and analysed in order to preclude the interference of factors that changes between different patients which might influence the Endo180 level. The results showed that the Endo180 levels in plasma from 21 breast cancer patients did not correlate with their pathological characteristics.

Table 5.11 Age composition of patients and corresponding Endo180 levels

	Early, n = 12		Advanced, n = 9	
	n (%)	Endo180	n (%)	Endo180
Age at sample collection				
<50	3 (25)	27.46 ± 4.46	0 (0)	-
50-59	4 (33)	35.23 ± 8.94	0 (0)	-
60-69	4 (33)	27.91 ± 2.43	5 (56)	46.55 ± 3.65
>70	1 (8)	27.78	4 (44)	33.64 ± 3.02

n (%): number of patients (percentage of patients). Endo180 levels (ng/mL): Mean ± SEM.

Table 5.11 shows the age distribution of the patients and its correlation with plasma Endo180 levels. The range of ages was 40 – 77 (median 58) for early breast cancer patients and 63 – 76 (median 67) for advanced breast cancer patients. To compare the correlation between age and Endo180 level, all early/advanced breast cancer patients were divided into four age-matched groups (<50, 50 – 59, 60 – 69, and ≥70). No statistical difference was found in Endo180 levels between different age groups in both early breast cancer patients (P = 0.694, Kruskal-Wallis test) and advanced breast cancer patients (P = 0.111, Mann-Whitney test). We also compared the difference in Endo180 level between early and advanced breast cancer patients that belong to the same age

group, and a significant statistical difference was shown in 60 – 69 group (P = 0.016, Mann-Whitney test), while other age groups were not comparable due to lack of samples.

Table 5.12 Hormone receptor status of patients and corresponding Endo180 levels

		Early, n = 62		Advanced, n = 18	
		n (%)	Endo180	n (%)	Endo180
ER					
	Negative	12 (19)	29.30 ± 1.86	3 (17)	72.72 ± 19.87
	Positive	47 (76)	34.77 ± 2.72	14 (78)	43.69 ± 3.27
	Not known	3 (4.8)	26.22 ± 1.83	1 (6)	47.58
PR					
	Negative	8 (13)	26.53 ± 2.01	5 (28)	61.78 ± 13.57
	Positive	6 (10)	42.81 ± 10.38	7 (39)	38.04 ± 3.33
	Not known	48 (77)	33.24 ± 2.36	6 (33)	50.37 ± 4.40
HER2					
	Negative	44 (71)	35.58 ± 2.82	11 (61)	53.06 ± 6.87
	Positive	10 (16)	25.10 ± 2.41	1 (5.6)	43.72
	Not known	8 (13)	31.00 ± 2.47	6 (33)	41.67 ± 4.75

n (%): number of patients (percentage of patients). Endo180 levels (ng/mL): Mean ± SEM.

The correlation between the hormone receptor status of breast cancer patients and the level of plasma Endo180 was also investigated. Because the hormone receptor status of all patients in cohort 1 and 2 were provided, we combined the patients in both cohorts and analysed them as one group. The composition and proportion of patients with different receptor status, and corresponding Endo180 levels are shown in Table 5.12. The statistical analysis result indicates no significant correlation between hormone receptor status with Endo180 level: 1) ER: no significant different between ER negative and ER positive samples within the whole group (P = 0.881, Mann-Whitney test), or within early breast cancer subgroup (P = 0.720, Mann-Whitney test) and advanced breast cancer subgroup (P = 0.197, Mann-Whitney test); 2) PR: no significant different between PR negative and PR positive samples within the whole group (P = 0.390, Mann-Whitney test), or within early breast cancer subgroup (P = 0.081, Mann-Whitney

test) and advanced breast cancer subgroup (P = 0.149, Mann-Whitney test); 3) HER2 Receptor: the difference between HER2 negative and HER2 positive samples was significant within the whole group (P = 0.038, Mann-Whitney test), but not within the early breast cancer subgroup (P = 0.065, Mann-Whitney test) and advanced breast cancer subgroup (P = 1, Mann-Whitney test). When comparing the difference in Endo180 levels between early breast cancer patients and advanced breast cancer patients with the same hormone receptor status, most of the subgroups showed a significant difference (ER negative: P = 0.004, Mann-Whitney test; ER positive: P = 0.009, Mann-Whitney test; PR negative: P = 0.006, Mann-Whitney test; PR positive: P = 0.836, Mann-Whitney test; HER2 negative: P = 0.004, Mann-Whitney test).

5.4.3 The correlation of plasma Endo180 with treatment status

Table 5.13 Treatment type and corresponding Endo180 levels in breast cancer patients

	Early, n = 62		Advanced, n = 18	
	n (%)	Endo180	n (%)	Endo180
Chemotherapy				
No	2 (3)	28.97 ± 4.20	5 (28)	59.23 ± 13.08
Yes	9 (15)	30.20 ± 4.17	9 (50)	45.35 ± 4.04
Not known	51 (82)	34.02 ± 2.46	4 (22)	43.29 ± 9.26
Hormonal therapy				
No	6 (10)	26.30 ± 3.26	5 (28)	59.88 ± 13.84
Yes	6 (10)	34.15 ± 5.15	11 (61)	43.88 ± 2.83
Not known	50 (80)	34.04 ± 2.51	2 (11)	47.65 ± 20.05
Bisphosphonates				
No	0 (0)	-	8 (44)	58.30 ± 8.79
Yes	2 (3)	29.59 ± 6.78	2 (11)	42.99 ± 0.73
Not known	60 (97)	33.42 ± 2.18	8 (44)	40.63 ± 3.65
Denosumab				
No	0 (0)	-	6 (33)	52.71 ± 11.83
Yes	0 (0)	-	11 (61)	48.11 ± 4.07
Not known	62 (100)	33.30 ± 2.11	1 (6)	31.96

n (%): number of patients (percentage of patients). Endo180 levels (ng/mL): Mean ± SEM.

The correlation between the treatment (chemotherapy, hormonal therapy, or bone therapy) status of breast cancer patients with plasma Endo180 levels was analysed. Patients in both cohort 1 and cohort 2 were combined and analysed as one group. Table 5.13 shows the classification of the treatments and the numbers of receiving or not receiving each treatment type. Results of the statistical analysis show no significant difference in terms of plasma Endo180 level between patients who have or have not received a specific treatment type: 1) Chemotherapy: no significant different between treated and untreated patients within the whole group ($P = 0.27$, Mann-Whitney test), or within early breast cancer subgroup ($P = 0.909$, Mann-Whitney test) and advanced breast cancer subgroup ($P = 0.518$, Mann-Whitney test); 2) Hormonal therapy: no significant different between treated and untreated patients within the whole group ($P = 0.547$, Mann-Whitney test), or within early breast cancer subgroup ($P = 0.458$, Mann-Whitney test) and advanced breast cancer subgroup ($P = 0.320$, Mann-Whitney test); 3) Bisphosphonates: no significant difference between treated and untreated patients within the whole group ($P = 0.109$, Mann-Whitney test), or within advanced breast cancer subgroup ($P = 0.533$, Mann-Whitney test); 4) Denosumab: no significant different between treated and untreated patients within advanced breast cancer subgroup ($P = 1$, Mann-Whitney test). When comparing the difference in Endo180 levels between early and advanced breast cancer patients within the same treatment type, most of the subgroups showed a significant difference (chemotherapy untreated: $P = 0.095$, Mann-Whitney test; chemotherapy treated: $P = 0.011$, Mann-Whitney test; hormonal therapy untreated: $P = 0.03$, Mann-Whitney test; hormonal therapy treated: $P = 0.048$, Mann-Whitney test; bisphosphonates treated: $P = 0.333$, Mann-Whitney test). Those with high P value (chemotherapy untreated, bisphosphonates treated) have limited number of samples to assure the reliability of the statistical analysis.

5.4.4 The significant difference in Endo180 level between early and advanced breast cancer patients

We then compared the range of Endo180 levels in the plasma of early breast cancer patients with that of advanced breast cancer patients, and found a significant difference between the two groups (P = 0.0001, Mann-Whitney test). The distributions of each group as is shown in Figure 5.2A: 1) Early breast cancer patients: n = 62, mean ± standard error = 33.30 ± 2.11, range = 12.37 – 56.31, Q_1 = 23.32, Q_2 = 29.63, Q_3 = 36.98, 95% confidence interval of mean: 29.07 – 37.53; 2) Advanced breast cancer patients: n = 18, mean ± standard error = 48.75 ± 4.57, range = 27.60 – 67.70, Q_1 = 38.18, Q_2 = 42.52, Q_3 = 59.77, 95% confidence interval of mean: 39.10 – 58.39 (Kolmogorov–Smirnov test). The plasma Endo180 level in advanced breast cancer patients was significantly higher than in early breast cancer patients. This indicates a correlation between high plasma Endo180 level with the advanced stage of breast cancer patients.

The plasma levels of other biomarkers and their correlation with the stage of breast cancer were also investigated. As is shown in Figure 5.2B, BALP showed no significant difference between early and advanced breast cancer patients (P = 0.486, Mann-Whitney test). As no data of CA 15-3 and CTX antigen levels in early breast cancer patients was provided, it was not possible to compare the difference in CA 15-3 and CTX levels between early and advanced breast cancer patients.

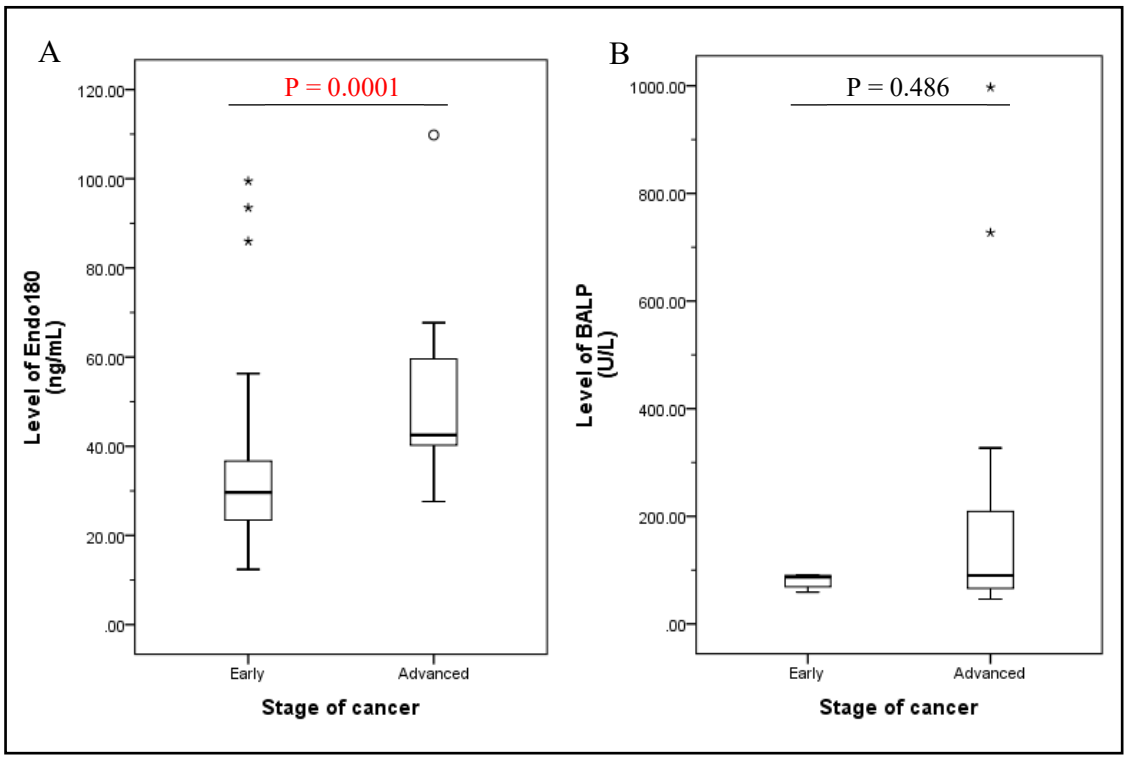


Figure 5.2 Comparison between early and advanced breast cancer patients in plasma Endo180 and BALP levels

Plasma Endo180 levels were measured using Endo180 ELISA, samples were subject to a 2-fold dilution before being analysed. The level of Endo180 was calculated by normalising the reading of each sample to that of the standard protein. BALP levels were measured by our collaborators at the University of Liverpool. The box-and-whisker plots show the total range, interquartile distribution, outliers (asterisks and open circles) and median value (horizontal line) of Endo180/BALP levels in the plasma of early breast cancer patients and advanced breast cancer patients respectively. Mann-Whitney test was used to test the significant difference in Endo180/BALP between early breast cancer patients (n = 62 for Endo180, n = 21 for BALP) and advanced breast cancer patients (n = 18 for Endo180, n = 5 for BALP), $P < 0.05$ was considered as significant difference.

The breast cancer patients were divided into subgroups according to the different types of metastatic sites, in order to investigate the correlation between metastatic sites and plasma Endo180 levels. Kruskal-Wallis test indicated that there was no significant difference (P = 0.262) in plasma Endo180 levels between bone metastasis (mean ± standard error = 36.98 ± 4.05, n = 9), lung metastasis (60.35, n = 1), bone + lung metastasis (mean ± standard error = 41.26 ± 1.00, n = 2), and bone + peritoneum metastasis (mean ± standard error = 55.01 ± 12.69, n = 2), liver metastasis (mean ± standard error = 61.24 ± 16.32, n = 4); bone + liver metastasis (66.53, n = 1), bone and liver metastasis with dissemination in brain (27.60, n = 1).

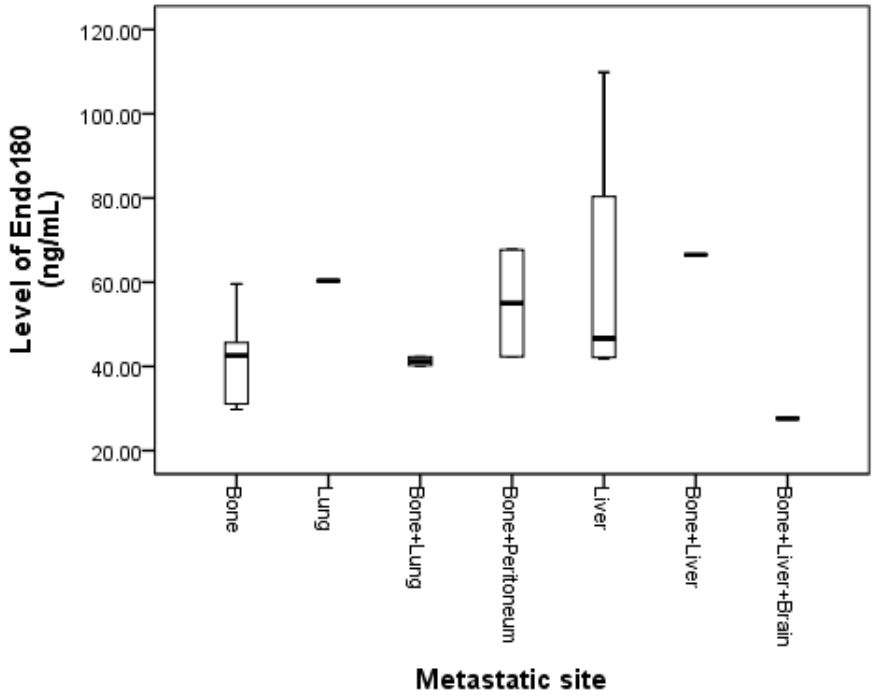


Figure 5.3 Comparison of plasma Endo180 levels in advanced breast cancer patients with different metastatic sites

The box-and-whisker plot described the range, interquartile distribution, outliers (asterisks) and median value (horizontal line) of plasma Endo180 levels in breast cancer patients with different metastatic sites. From left to right: bone metastasis alone (n = 7), lung metastasis alone (n = 1), bone and lung metastasis (n = 2), bone and peritoneum metastasis (n = 2), liver metastasis alone (n = 4); bone and liver metastasis (n = 1), bone and liver metastasis with dissemination in brain (n = 1).

5.4.5 Plasma Endo180 as a diagnostic indicator of advanced breast cancer

5.4.5.1 Evaluation of diagnostic accuracy and determination of cut-off value

In order to use plasma Endo180 as a predictive tool for advanced breast cancer, a cut-off value needs to be found out to determine the positive and negative results in the assay. We first analysed the distribution of plasma Endo180 levels in out samples from 62 early breast cancer patients and 18 advanced breast cancer patients. The 95% CI shows an estimation on the distribution of Endo180 levels in early and advanced breast cancer patients respectively (Figure 5.4).

ROC curve analysis was then used to evaluate the diagnostic accuracy of plasma Endo180 in predicting advanced breast cancer. The result showed that Endo180 is a sensitive marker in reflecting the occurrence of advanced breast cancer (area under the curve: 0.746 ± 0.062, P = 0.001). The level of plasma Endo180 at 40.06 ng/ mL was

selected as the best cut-off value in the evaluation, with 70% sensitivity and 81% specificity (Figure 5.5).

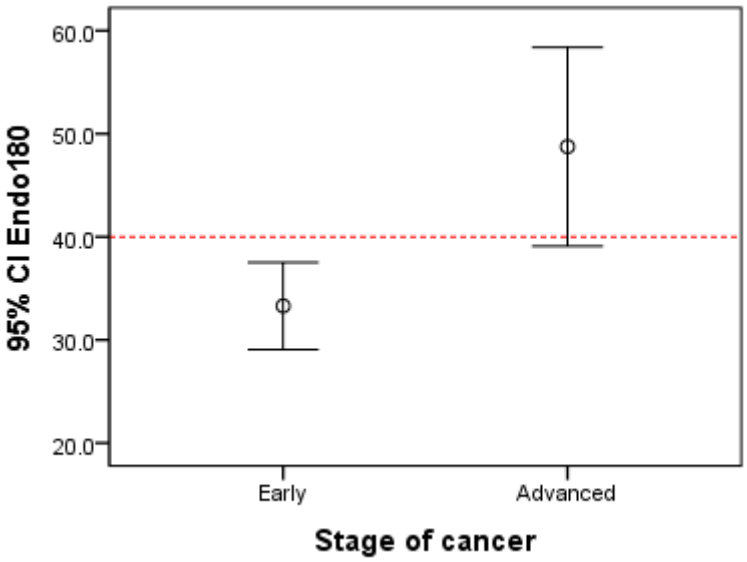


Figure 5.4 Distribution of Endo180 levels in early and advanced breast cancer patients

This figure summarises the distribution of the values of plasma Endo180 levels in early breast cancer patients (n = 62) and advanced breast cancer patients (n = 18) respectively. The error bars show the upper and lower bounds of 95% confidence interval of mean (95% CI) of each group – early breast cancer patients: 29.07 – 37.53 ng/mL, advanced breast cancer patients: 39.10 – 58.39 ng/mL.

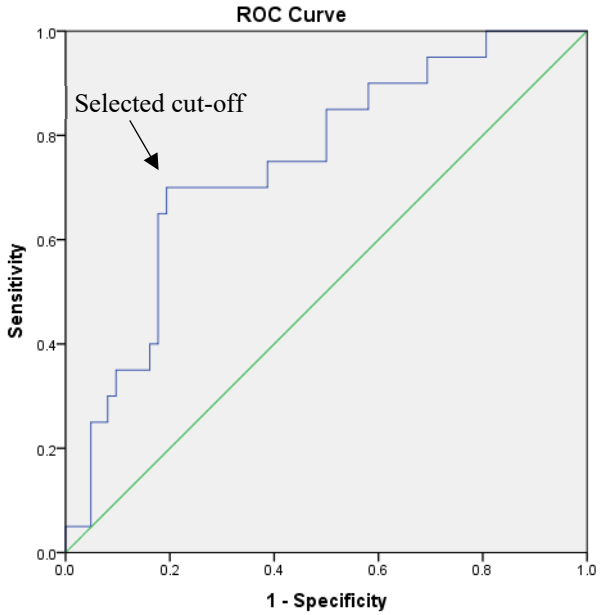


Figure 5.5 ROC curve for plasma Endo180 to distinguish advanced breast cancer

Two criterions were used in the evaluation: 1) sensitivity (true positive rate) was defined as the proportion of true positive samples (advanced breast cancer patients with Endo180 levels higher than the cut-off value) in all advanced breast cancer patients, which represents the capability of Endo180 in predicting advanced breast cancer cases; 2) 1 - specificity (false positive rate) was defined as the proportion of false positive samples (early breast cancer patients Endo180 levels higher than the cut-off value) in all early breast cancer patients, which represents the capability of Endo180 in distinguishing advanced breast cancer with early breast cancer. Note: purple line – ROC curve for Endo180, green line – reference line.

5.4.5.2 Changes following treatments

There were two advanced breast cancer patients who had a second plasma collection ten months after the first sample collection. We measured and compared the plasma Endo180 in the two samples from the same patients, which represented the level of Endo180 before and after treatment. The result from both patients showed a decrease of in Endo180 (16% – 30%) in the second samples in comparison to the first samples (Figure 5.6). Patient 1 received chemotherapy and hormonal therapy, while patient 2 received chemotherapy and bone therapy (denosumab). This indicates that the level of plasma Endo180 might be able to reflect the effect of some types of anti-tumour therapy, or patients' response to specific treatments. However, due to the lack of enough samples and information, a complete statistical analysis could not be performed regarding the correlation between plasma Endo180 and treatment effect.

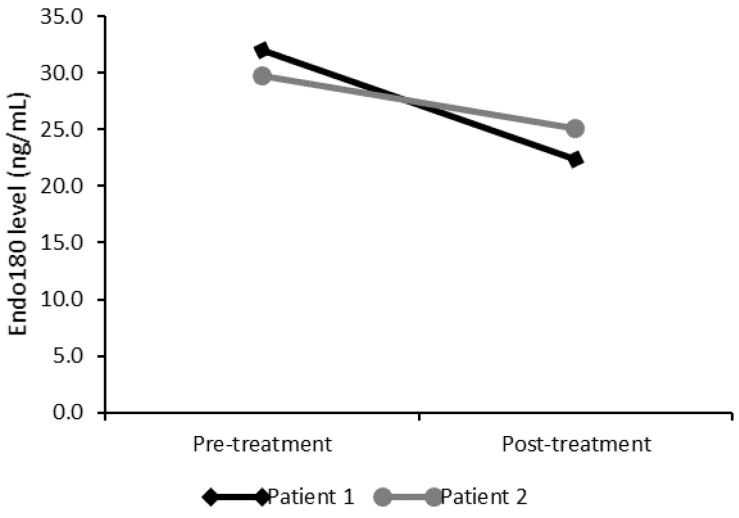


Figure 5.6 change of Endo180 levels following treatment

Plasma samples collected before and after treatments from the same patient (n = 2) were used to investigate the modulation of Endo180 levels by the treatment. The figure shows the change of plasma Endo180 levels in the two advanced breast cancer patients before and after treatment.

5.4.6 Comparison of Endo180 with conventional tumour markers

5.4.6.1 Correlation with advanced breast cancer

Besides the level of Endo180 being measured in this study, the levels of three conventional biomarkers, BALP, CA 15-3 and CTX were also measured in the patient samples. The levels of these biomarkers were measured by our collaborators at the University of Liverpool. Only limited number of the patient samples had these biomarkers measured, including: BALP was measured in 26 samples (5 samples from early breast cancer patients and 21 samples from advanced breast cancer patients), CA 15-3 was measured in 12 samples from advanced breast cancer patients, and CRX was measured in 5 samples from advanced breast cancer patients. The cut-off values for other biomarkers were used as described in previous studies: CA 15-3 – 28 U/mL; BALP – 140 U/L; CTX – 4.5 ng/mL (Nabeya et al., 2002, Ahmed and Gibbons, 2015, Shao et al., 2015). The result showed that Endo180 had a higher true positive proportion (78%) in comparison to CA 15-3 (67%) and CTX (0%) in correlation with advanced breast cancer.

5.4.6.2 Linear correlation between Endo180 and other tumour markers

Besides the comparisons mentioned above, we also performed linear regression analyses using patient samples that have at least two different biomarkers measured (Endo180 and CA 15-3, or Endo180 and BALP, or Endo180 and CTX), in order to investigate the linear relationship between plasma Endo180 level and other biomarkers (Figure 5.7). Correlation test was also applied to inspect the effectiveness of the regression analysis. According to the result, Endo180 shows a strong linear correlation with CTX ($r^2 = 0.984$, $P = 0.008$ in linear regression analysis; $P < 0.01$ in Spearman correlation test), a linear correlation with CA 15-3 ($r^2 = 0.605$, $P = 0.014$ in linear regression analysis; $P = 0.036$ in Spearman correlation test), and no linear correlation

with BALP (r^2 = 0.469, P = 0.0004 in linear regression analysis; P = 0.016 in Spearman correlation test).

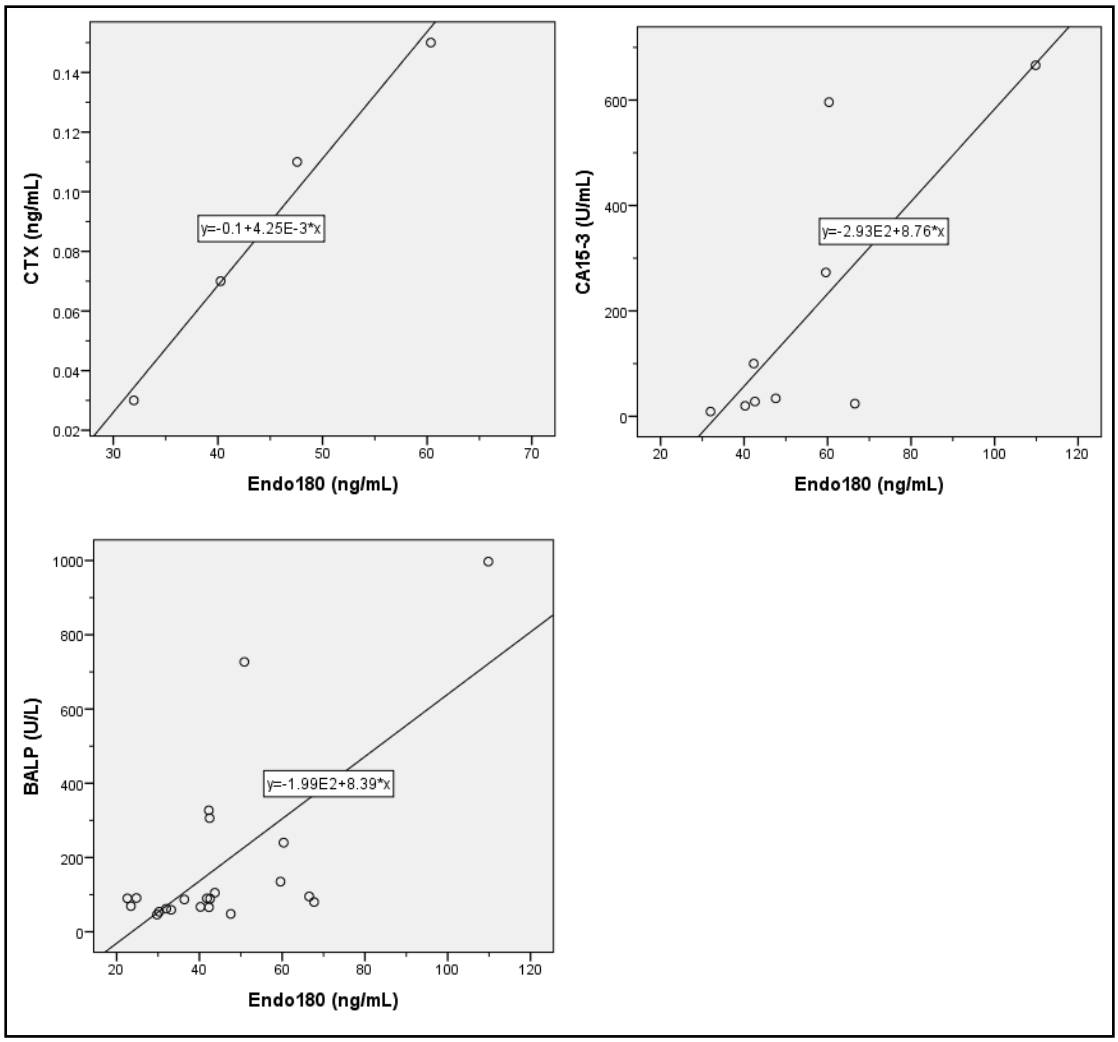


Figure 5.7 Linear regression between Endo180 and other biomarkers

Patient samples with Endo180 and another biomarker (CA15-3, BALP or CTX) measured were selected to analyse the linear correlation between the markers. The outlier samples (samples that has the measurement output of a biomarker extremely higher than the rest of samples) were removed before the assay was performed. The scatters above show the distribution relationship between Endo180 and each specific biomarker, and the fit line indicates a linear regression analysis between the two biomarkers analysed. The coefficient of determination (r^2) for each fit line is as below: Endo180 and CA15-3, r^2=0.605; Endo180 and BALP, r^2=0.469; Endo180 and CTX, r^2=0.984.

5.5 Discussion

The value of Endo180 as a cancer biomarker is evident from its elevated expression in tumour tissue including breast, prostate, glioblastoma multiforme, head and neck, and liver, being linked to clinical grade, disease progression and/or decreased survival (Sulek et al., 2007, Wienke et al., 2007, Kogianni et al., 2009, Huijbers et al., 2010, Gai et al., 2014, Rodriguez-Teja et al., 2015a, Rodriguez-Teja et al., 2015b). Recent study showed that Endo180 is released by breast cancer cells MDA-MB-231 and MCF7-E, and could be detected in the blood (Palmieri et al., 2013a). In this study, the data collected by analysing the plasma samples from breast cancer patients shows that Endo180 levels are significantly different between early and advanced breast cancer patients (Figure 5.2). This indicates that the plasma Endo180 level in breast cancer patients is correlated with the stage of disease, and Endo180 has the potential to be used as a tumour marker for advanced breast cancer.

Increased ectodomain shedding of Endo180 could occur before or after the tumour cells have migrated and disseminated to secondary sites in the bone and/or visceral tissues. Based on previous findings that Endo180 is dysregulated in a prostate cancer model of bone metastasis, it is plausible that the increased Endo180 level in advanced breast cancer results from its bidirectional regulation at the tumour-stromal interface via a cleavage mechanism that leads to its release from tumour cells and/or bone cells (Caley et al., 2012). This led to the further *in vitro* study into the effect of the interaction between tumour cells and osteoblasts as described in Chapter 7.

Endo180 has cooperative functions with uPA/uPAR and MT1-MMP/MMP-2 in collagen remodelling and invasive cell behaviour (Sturge et al., 2003, Behrendt, 2004, Madsen et al., 2007, Wagenaar-Miller et al., 2007, Kogianni et al., 2009, Ye et al., 2010, Madsen et al., 2013). In combination with the fact that the expression of Endo180 is

modulated during the progression of bone metastasis, it is possible that the increase of plasma Endo180 in patient plasma was released by the invasive tumour cells, which means that there might be a correlation between the occurrence of invasive tumour cells (EMT of tumour cells at the primary site) and the increased level of Endo180 released into the circulation (Caley et al., 2012).

Endo180 plays an important role in collagen uptake and ECM turnover during tumour cell migration, and is proved to cooperate with MT1-MMP in collagen degradation procedures (Curino et al., 2005, Madsen et al., 2007, Wagenaar-Miller et al., 2007, Ye et al., 2010). In this study, a correlation in the serum/plasma levels between Endo180 and some other conventional tumour markers for breast cancer was observed. The release of the extracellular domain of the MUC-1 protein into the ECM makes it possible to measure the serum level of CA 15-3. This cleavage is proved to be mediated by protease MT1-MMP (Thathiah and Carson, 2004). The co-functional relationship between Endo180 and MT1-MMP suggests a similar release mechanism and a correlation in expression and function between Endo180 and CA 15-3. As an endocytic recycling glycoprotein of the mannose receptor family, Endo180 is related to collagen uptake and deposition, and is proved to have the capability of binding to the C-terminal region of type I collagen (Mousavi et al., 2005, Thomas et al., 2005). As CTX is enzymatic hydrolysis products of type I collagen containing cross-linking regions, it is supposed that a correlation exists between the release of End180 and CTX, and Endo180 could influence the level of CTX via the regulation of collagen degradation. Also, the function and characteristics of Endo180 make it a collagen-related biomarker, which is different from BALP, a marker of bone formation (Garnero and Delmas, 1993). This could explain the uncorrelation between plasma Endo180 and BALP levels in breast cancer patients.

The major limitation of this study was the small sample size. The relatively small sample size made the sample less representative to the whole patient population, which means that the results could be more significantly influenced by the individual characteristic of a patient. Moreover, the incomplete information provided by our collaborator resulted in less samples available for the statistical analysis. For example, when comparing the plasma Endo180 levels among patients in different age groups, we could only use the samples from 9 advanced breast cancer patients and 12 early breast cancer patients, because no age information of the 59 patients in cohort 2 was provided (Table 5.11). Similar situation existed in the statistical analysis to investigate the difference in plasma Endo180 between patients with/without a specific treatment type. Due to the incomplete treatment information, most of the patients ended up in the "unknown" section, which means that they could not be used in the comparison (Table 5.13). Moreover, the lack of survival outcome / current status of the patients in cohort 2 meant that there were only 5 patients available for the survival analysis, which made it impossible to carry out a proper statistical analysis. As to the measurement of conventional biomarker, only very few of the samples had CA 15-3 (n = 12), BALP (n = 26) and CTX (n = 5) measured. A comparison between Endo180 and these biomarkers was impossible with such a small sample size. As a result, only a limited number of samples in cohort 1 could be used in the statistical analysis. In order to produce convincing results, a larger sample size with full related information need to be properly scheduled and obtained for future investigations.

Due to the limited number and type of breast cancer patients in this study, only preliminary results could be given regarding the potential of plasma Endo180 as a biomarker for advanced breast cancer. Moreover, the lack of following-up sample collection and information of the same patients, especially those early breast cancer

patients with high initial Endo180 levels, made it impossible to validate the capability of Endo180 in predicting the progress of tumour stage and the poor prognosis of those patients. A further study that contains a larger and more completed patient group (including early/advanced breast cancer patients at different stages of disease, and at different time points before/after treatment) will be able to provide a deeper understanding of how the plasma Endo180 level changes during the progression of cancer, as well as to further validate Endo180 as a marker for advance breast cancer, or metastatic bone disease that is related to different kinds of solid tumours.

The effect of bisphosphonates in the release of Endo180 is another research question that needs to be investigated. Recent studies showed that adjuvant bisphosphonate treatment could reduce the rate of breast cancer recurrence in the bone and improve breast cancer survival of postmenopausal breast cancer patients (Early Breast Cancer Trialists' Collaborative Group, 2015). Given the preliminary results in our previous study which showed that Endo180 release is suppressed by bisphosphonates, it is useful to determine if bisphosphonates modulate the expression/release of Endo180 in breast cancer or other type with bone metastasis, and the mechanism behind this modulation. Investigation could also be carried out to find out how plasma Endo180 level changes throughout the bisphosphonate treatment (Palmieri et al., 2013a).

The expression of Endo180 is also found to be correlated with prostate cancer progression (Kogianni et al., 2009). Besides breast cancer and prostate cancer, it will be of interest to explore the role of Endo180 as a more general marker of cancer progression in multiple cancer types, including the fore-mentioned primary cancer affecting visceral tissues (glioblastoma multiforme, head and neck, liver) and primary bone cancers such as osteosarcoma (Engelholm et al., 2016, Sturge, 2016).

6 The effect of bisphosphonates on Endo180 release and expression

6.1 Overview

Bone metastasis is the most common secondary cancer that derives from *in situ* primary carcinomas in breast, prostate and lung cancer (Mundy, 2002, Valastyan and Weinberg, 2011). Patients with osteolytic metastatic bone disease would usually receive anti-resorption treatments, such as bisphosphonates and denosumab (Neville-Webbe et al., 2010). As discussed in Section 2.5.2.3, the recent analysis of data obtained from several collaborative clinical trials showed that adjuvant bisphosphonate treatment in early breast cancer could reduce the rate of breast cancer recurrence in the bone, and improves survival outcomes in postmenopausal breast cancer patients (Early Breast Cancer Trialists' Collaborative Group, 2015).

Bisphosphonates are widely used in breast or prostate cancer patients with bone metastasis, and the level of Endo180 in the plasma of breast cancer patients was shown to be modulated by bisphosphonates (Palmieri et al., 2013a). Therefore, we hypothesised that the expression and release of Endo180 from breast cancer cells and osteoblasts are regulated by bisphosphonates, which might be one of the anti-tumour effects of bisphosphonates. The aim of this chapter was to use *in vitro* experiments to investigate the regulation of Endo180 expression and release in tumour cells and osteoblasts under bisphosphonate treatment. The results indicate that the expression and release of Endo180 in breast cancer cell line MDA-MB-231 and prostate cancer cell line PC-3 are inhibited by bisphosphonate treatment, while those in osteoblasts and osteoblast-like cells were not significantly altered.

6.2 Experimental setup

In order to investigate the effect of bisphosphonates on the release and expression of Endo180 in tumour cells and osteoblasts, and to understand the mechanism underlying the anti-tumour effect of bisphosphonates on breast cancer and metastatic bone disease, breast cancer cells, human osteoblasts and osteoblast-like cells were cultured in vitro and treated with bisphosphonates. Two types of bisphosphonates, alendronic acid and zoledronic acid, were used in this study. After treatment, conditioned medium was collected and analysed using the Endo180 ELISA, to measure the level of Endo180 released into the medium. Cell lysate was analysed using Western blot to measure Endo180 expression level. Living cells were stained with Endo180 antibody and analysed using flow cytometry, to measure the level of Endo180 on cell surface. The comparison of Endo180 levels between treated cells and untreated cells were carried out to determine the effect of bisphosphonates on Endo180 release and expression (Figure 6.1).

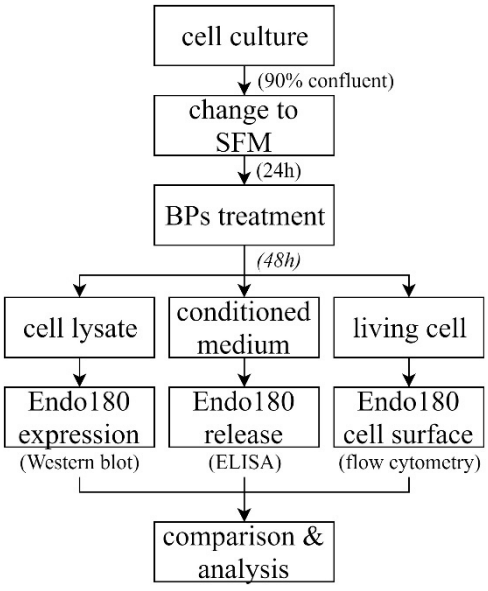


Figure 6.1 Design of in vitro bisphosphonate treatment experiments
Note: SFM – serum free medium, BPs – bisphosphonates (alendronic acid or zoledronic acid).

The concentration range of bisphosphonates used in the treatments were 3 – 100 μM. When determining the concentrations of bisphosphonates used in the treatment, we first considered the actual concentration of drugs in the effective sites of the patient. The administration dosage of each drug is a continuous 10 mg daily oral dose for alendronic acid, and 4 mg per intravenous infusion every 3 – 4 weeks for zoledronic acid. However, it was difficult to quantify the absorption of drugs and their distribution to different parts of the body. Even when focused only on bone, the knowledge about the distribution of bisphosphonates is limited (Cremers and Papapoulos, 2011). It has been showed that bisphosphonates are not evenly distributed throughout skeletons (Carnevale et al., 2000), and the retention of drug differs (as much as a three-fold difference) between patients (Chen et al., 2002). Moreover, the accumulation of bisphosphonates and the amount that will retain in the skeleton due to the relatively long retention of the drug also need to be considered. For this reason, it is difficult to determine the actual drug concentrations that cells are exposed to *in vivo*. Therefore, the bisphosphonate treatment concentrations used in this study were based on biologically effective concentrations used in previous studies (van der Pluijm et al., 1996, Aparicio et al., 1998, Derenne et al., 1999, Jagdev et al., 2001, Lee et al., 2001, Alakangas et al., 2002, Senaratne et al., 2002, Tassone et al., 2003, van Beek et al., 2003, Pan et al., 2004, Verdijk et al., 2007, Xiong et al., 2009, Tatsuda et al., 2010, Thiele et al., 2011, Wilke et al., 2014, Wilson et al., 2015), the details of which are presented in Table 2.3.

MDA-MB-231, MCF-7, MCF7-E, MCF7-D, PC-3, RWPE-1, U-2 OS and human osteoblasts (hOB) were recovered and cultured as described in Section 3.2.1. Cells were detached and seeded into 6-well plates at a concentration of 2×10^5 cells/well once they were sub-confluent in T75 tissue culture flasks. Cells were allowed to adhere and

expand for 24 hours before being starved in serum-free medium for another 24 hours. Then the conditioned media containing different concentrations of bisphosphonates was added to the treatment groups. Control groups were treated with same volumes of conditioned medium without bisphosphonates.

After 48 hours, the medium was collected, centrifuged at 12,000 rpm, 4℃ for 10 minutes. The level of Endo180 in the collected conditioned medium was measured using the Endo180 ELISA developed in this study as described in Section 3.2.6.2. To measure the level of Endo180 expression, the cell monolayer was trypsinaised, collected, and the centrifuged at 4℃, 1,000 rpm for 3 minutes, lysed as described in Section 3.2.3, and then analysed using Western blot as described in Section 3.2.8. To measure the level of Endo180 on cell surface, live cells were harvested and analysed using flow cytometry as described in Section 3.2.9.

6.3 Results

6.3.1 Bisphosphonates have different effects on Endo180 release in tumour cells and osteoblasts

The level of Endo180 released from tumour cells and osteoblastic cells under normal growth condition were first analysed by measuring Endo180 in the growth medium using ELISA (Figure 6.2). The results showed that breast cancer cells (MDA-MB-231, MCF-7) and prostate cancer cells (PC-3) have similar Endo180 release levels. MCF-7 cells that overexpress Endo180 (MCF7-E) release around 3-fold more Endo180 than un-transfected MCF-7 cells, and is at an analogous level of human osteoblasts and osteoblast-like cell line U-2 OS.

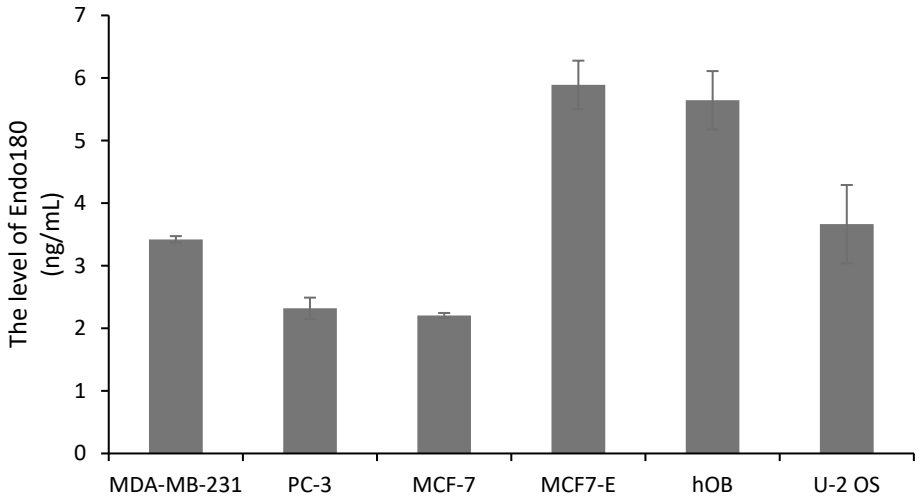


Figure 6.2 The levels of Endo180 released from different cell lines
Cells were seeded into 6-well plates at a concentration of 2×10^5 cells/well and were allowed to expand until ~90% confluent. Then cells were washed with PBS and 2 mL fresh growth medium was added. The release of Endo180 were assessed by measurement of Endo180 in the conditioned medium collected after 48 hours using ELISA. Results were represented as mean ± SEM (error bar), n = 2–6.

In order to investigate the regulatory effect of bisphosphonates on Endo180 release, we then analysed the level of Endo180 in the conditioned medium from cells treated with bisphosphonates. Cells without treatment under the same growth condition were used as control, and the relative Endo180 levels were calculated by normalising Endo180 concentration of the treatment samples with that of the control (Endo180 level set as 1). Results are shown in Figure 6.3 & 5.3 and described below:

(1) MDA-MB-231: Alendronic acid shows an inhibitory effect on the release of Endo180. This suppression is dose-dependent, with an inhibition of 37.4% – 58.3% at different treatment concentrations in comparison to control cells. Zoledronic acid also had an inhibitory effect at lower treatment concentrations (3 – 30 μM), but a dose-dependent effect was not observed. The results for 100 μM treatment from three repetitions were variable and showed no significant change. (Figure 6.3-A, B)

(2) MCF7-E: both alendronic acid and zoledronic acid significantly promoted the release of Endo180, with an upregulation of 14.5% – 205.2% (alendronic acid) and 9.7%

– 56.7% (zoledronic acid). The simulative effects were dose-dependent under both treatment types. The upregulation level changes gradually under zoledronic acid treatment, whereas a rapid increase occurred at 100 μM of alendronic acid. (Figure 6.3-C, D)

(3) Human osteoblasts: Alendronic acid promoted the release of Endo180 in comparison to control cells. This stimulation appears to be dose-dependent, but the analysis of variance showed a P value that is higher than 0.05. Zoledronic acid also stimulate the release of Endo180 at lower treatment concentrations (3 – 10 μM), and at 100 μM, a down regulation of Endo180 release was observed. However, the changes in all treatment groups were not statistically significant (P>0.05 in t-test), so we concluded that bisphosphonates have no significant effect on the release of Endo180 in osteoblasts (Figure 6.4-A, B)

(4) U-2 OS: under bisphosphonate treatment, the level of Endo180 released from U-2 OS cells showed an increase. However, the effect was not statistically significant (P > 0.05 in t-test), which lead to the conclusion that bisphosphonates have no significant effect on the release of Endo180 in U-2 OS cells (Figure 6.4-C, D)

Besides the cell types mentioned above, we also explored the effect of bisphosphonates on the release of Endo180 from other cell lines, including MCF-7 and MCF7-D (breast cancer cells), PC-3 (prostate cancer cells), RWPE-1 (prostate epithelial cells). In PC-3 cells, alendronic acid shows a significant dose-dependent suppression on the release of Endo180, and zoledronic acid shows no significant effect to Endo180 release (Figure 6.5 A, B); In MCF-7 cells, alendronic acid shows a dose-dependent inhibitory effect on the release of Endo180, and zoledronic acid also inhibits the release of Endo180 (Figure 6.5 C, D). As only two repetitions were done for each treatment, it was not possible to analyse the variance between different treatment groups in MCF-7 cells. However, the

consistent results in each repetition in each treatment groups suggests a significant suppression effect of bisphosphonates in MCF-7 cells. In MCF7-D and RWPE-1 cells, no significant regulation on the release of Endo180 was observed under the treatment of bisphosphonates (Figure 6.6).

From the results obtained in this study, we concluded that: 1) Endo180 is released from tumour cells and osteoblasts under normal growing conditions, and the release level is much higher in osteoblastic cells than breast / prostate cancer cells; 2) bisphosphonates could regulate the release of Endo180 from breast and prostate tumour cells, as well as osteoblasts. In breast cancer cell MDA-MB-231, the release of Endo180 is inhibited by bisphosphonates, while in osteoblasts, bisphosphonates have a promotive effect on Endo180 release; 3) in different cell lines, alendronic acid and zoledronic acid have different effectiveness on regulating the release of Endo180; 4) the release of Endo180 also increased in breast cancer cells that overexpress wild-type Endo180 under bisphosphonate treatment.

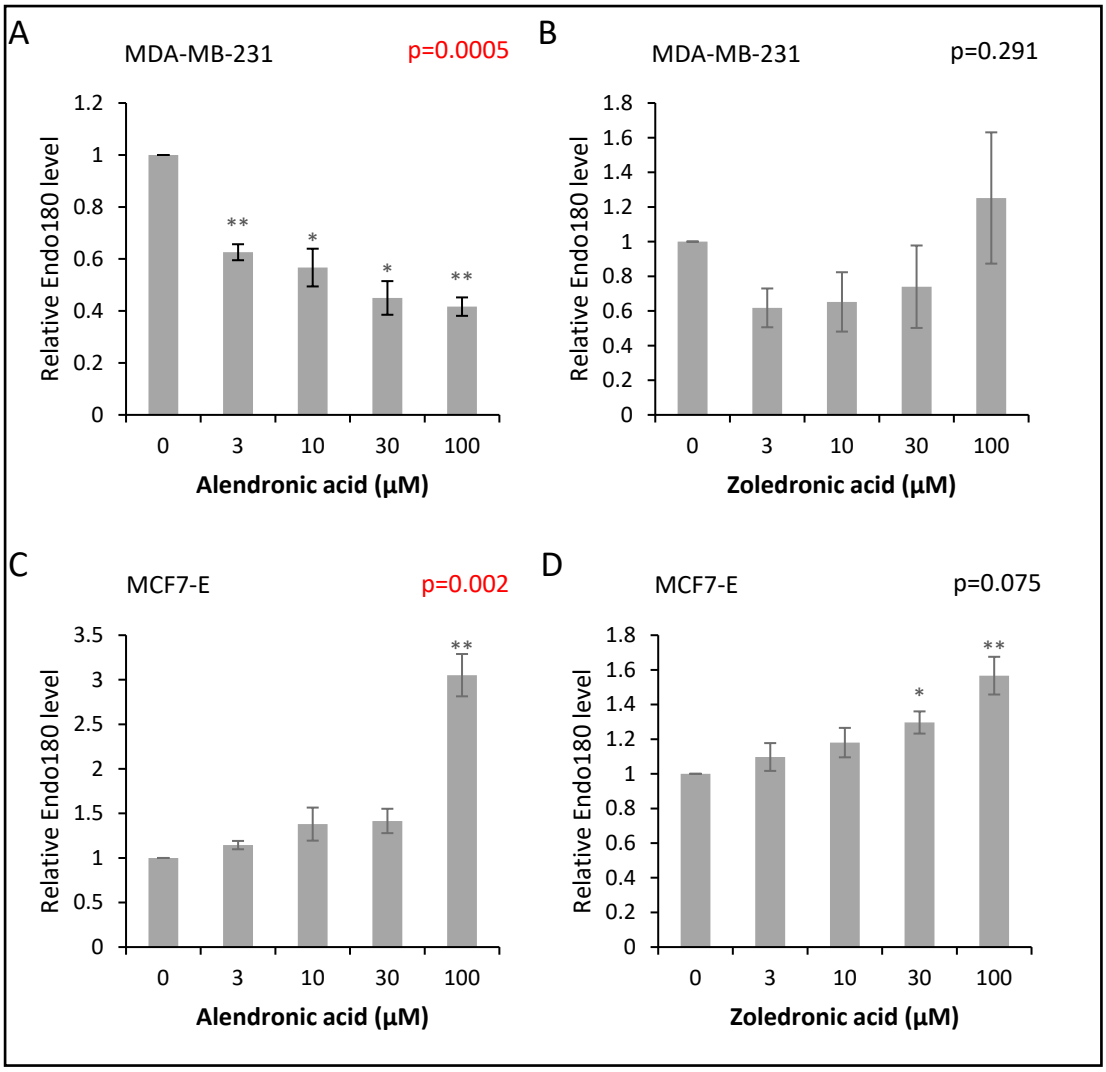


Figure 6.3 ELISA analysis on Endo180 levels in the conditioned medium from MDA-MB-231 and MCF7-E cells treated with bisphosphonates

MDA-MB-231 cells (A, B) and MCF7-E cells (C, D) were treated in serum-free conditioned medium that contains 3 – 100 µM of alendronic acid or zoledronic acid. After 48 hours, conditioned medium was collected and analysed using Endo180 ELISA. Relative Endo180 levels were compared by normalising the level of Endo180 in each treatment group to the corresponding control group (Endo180 level set as 1). Results were represented as mean ± SEM, n = 3 – 9, one-way ANOVA were used to test the significance of differences between groups (where ANOVA is not applicable due to poor variance homogeneity, nonparametric Kruskal-Wallis test was used as a replacement). $P \leqslant 0.05$ was considered as significant difference (marked in red). Student's t-test was used to compare the significance of the change in a specific treatment group with the control, * $P \leq 0.05$, ** $P \leq 0.01$.

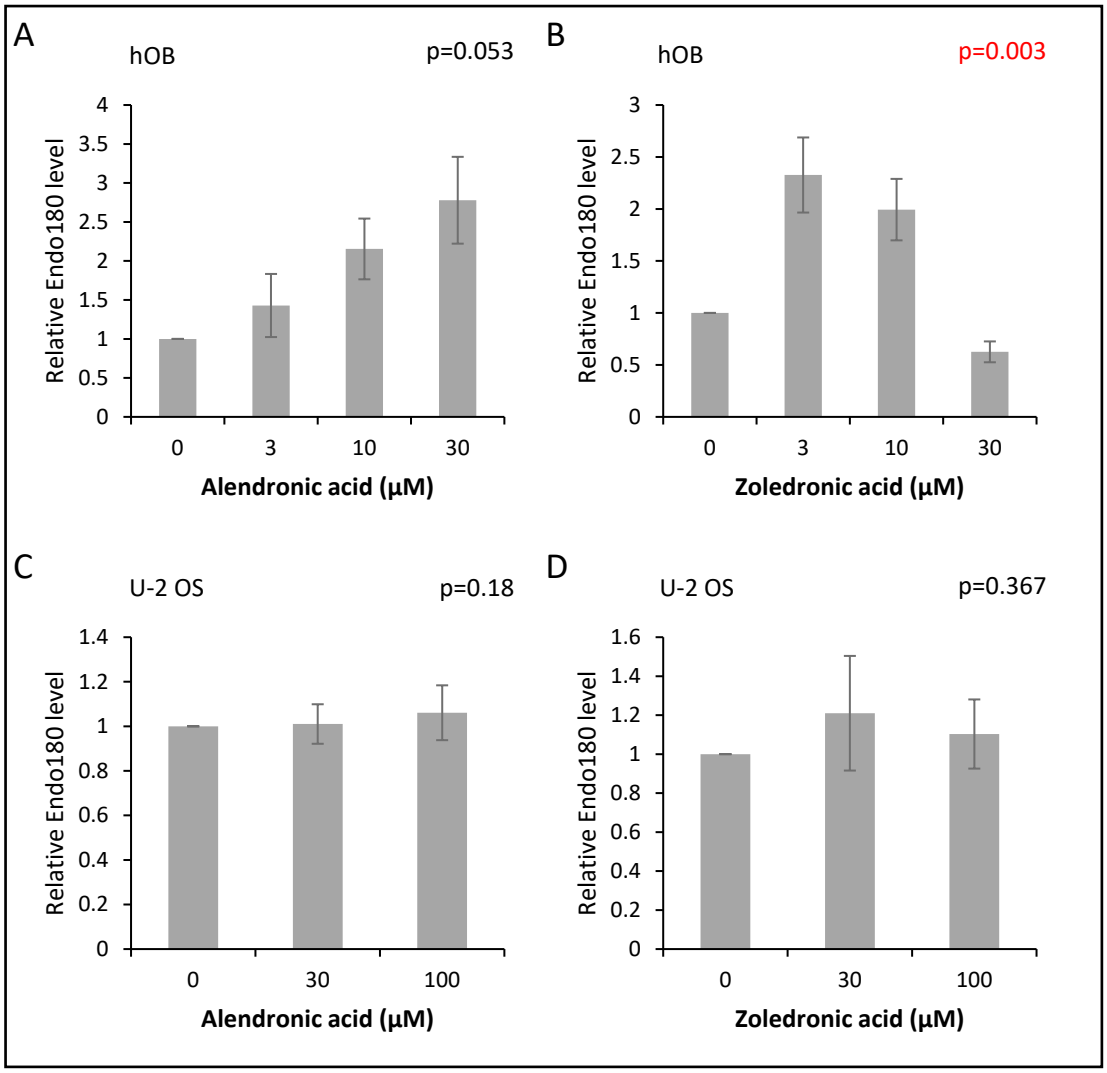


Figure 6.4 ELISA analysis on Endo180 levels in the conditioned medium from osteoblasts and U-2 OS cells treated with bisphosphonates

Osteoblasts were treated with 3 – 30 µM alendronic acid (A) or zoledronic acid (B). U-2 OS cells were treated with 30 – 100 µM alendronic acid (C) or zoledronic acid (B). After 48 hours, conditioned medium was collected and analysed using Endo180 ELISA. Relative Endo180 levels were compared by normalising Endo180 level of each treatment group to the corresponding control group (Endo180 level set as 1). Results were represented as mean ± SEM, n = 3, one-way ANOVA were used to test the significance of differences between groups (where ANOVA is not applicable due to poor variance homogeneity, nonparametric Kruskal-Wallis test was used as a replacement). $P \leqslant 0.05$ was considered as significant difference. Student's t-test was used to compare the significance of the change in a specific treatment group with the control.

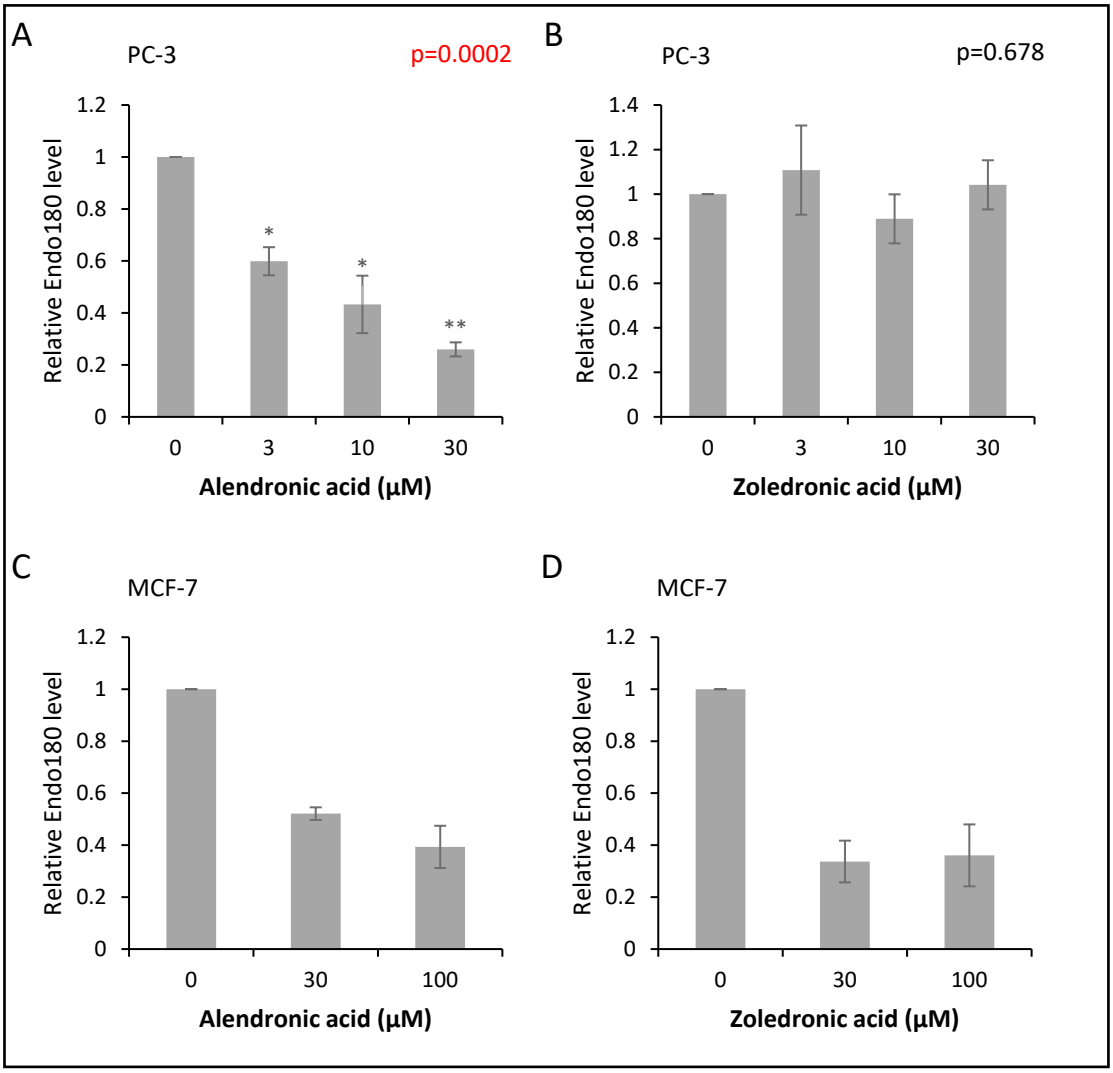


Figure 6.5 ELISA analysis on Endo180 levels in the conditioned medium from PC-3 and MCF-7 cells treated with bisphosphonates

PC-3 cells (A, B) and MCF-7 cells (C, D) were treated in serum-free conditioned medium that contains 30–100 µM of alendronic acid or zoledronic acid. After 48 hours, conditioned medium was collected and analysed using Endo180 ELISA. Relative Endo180 levels were compared by normalising the level of Endo180 in each treatment group to the corresponding control group (Endo180 level set as 1). Results were represented as mean ± SEM, n = 2 – 3, one-way ANOVA were used to test the significance of differences between groups (where ANOVA is not applicable due to poor variance homogeneity, nonparametric Kruskal-Wallis test was used as a replacement). $P \leqslant 0.05$ was considered as significant difference (marked in red). Student's t-test was used to compare the significance of the change in a specific treatment group with the control, * $P \leq 0.05$, ** $P \leq 0.01$.

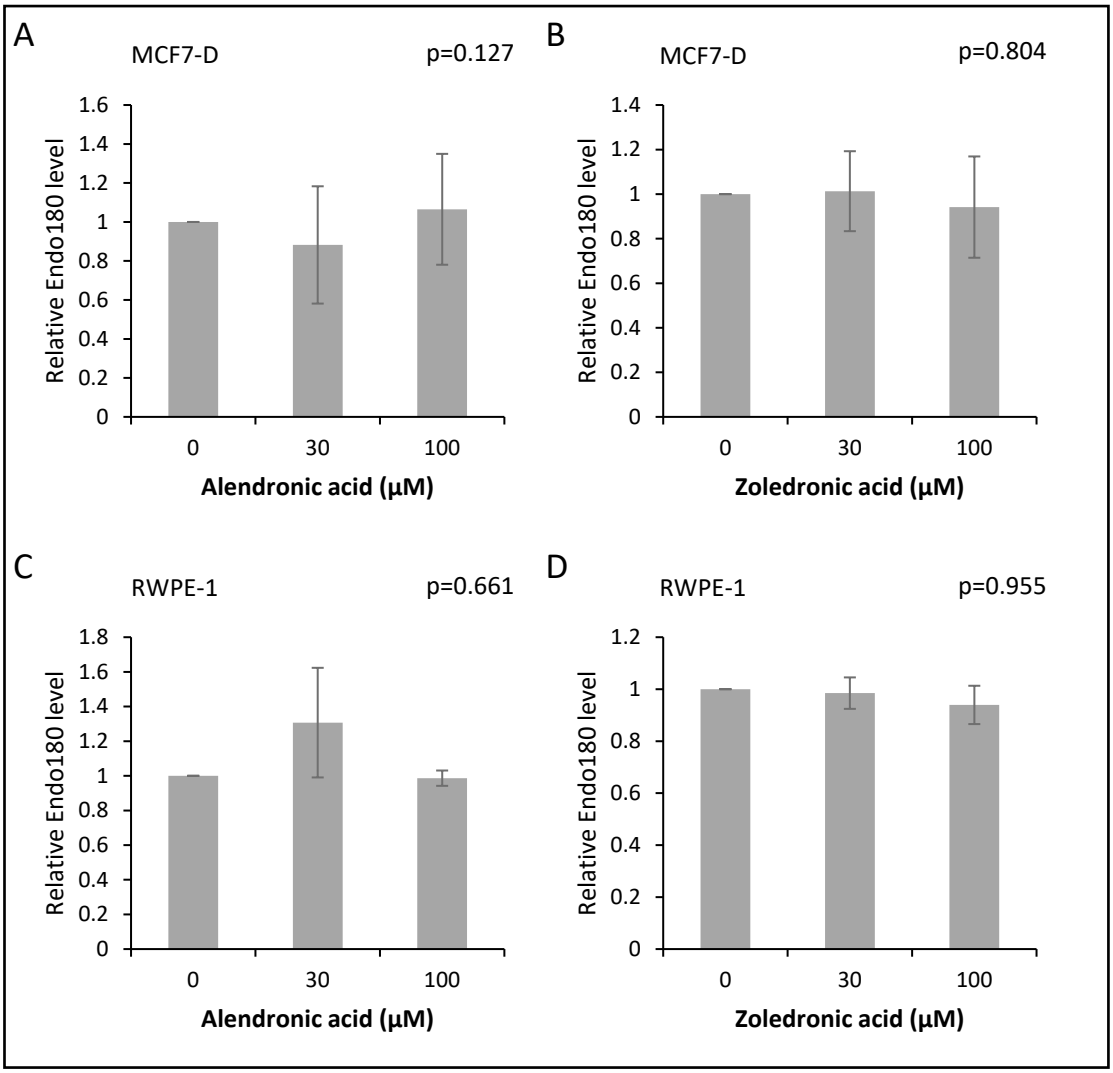


Figure 6.6 ELISA analysis on Endo180 levels in the conditioned medium from MCF7-D and RWPE-1 cells treated with bisphosphonates

MCF7-D (A, B) and RWPE-1 (C, D) cells were treated in serum-free conditioned medium that contains 30–100 µM of alendronic acid or zoledronic acid. After 48 hours, conditioned medium was collected and analysed using Endo180 ELISA. Relative Endo180 levels were compared by normalising the level of Endo180 in each treatment group to the corresponding control group (Endo180 level set as 1). Results were represented as mean ± SEM, n = 3, one-way ANOVA were used to test the significance of differences between groups (where ANOVA is not applicable due to poor variance homogeneity, nonparametric Kruskal-Wallis test was used as a replacement). $P \leqslant 0.05$ was considered as significant difference (marked in red). Student's t-test was used to compare the significance of the change in a specific treatment group with the control.

6.3.2 Bisphosphonates inhibit Endo180 expression in breast cancer cells

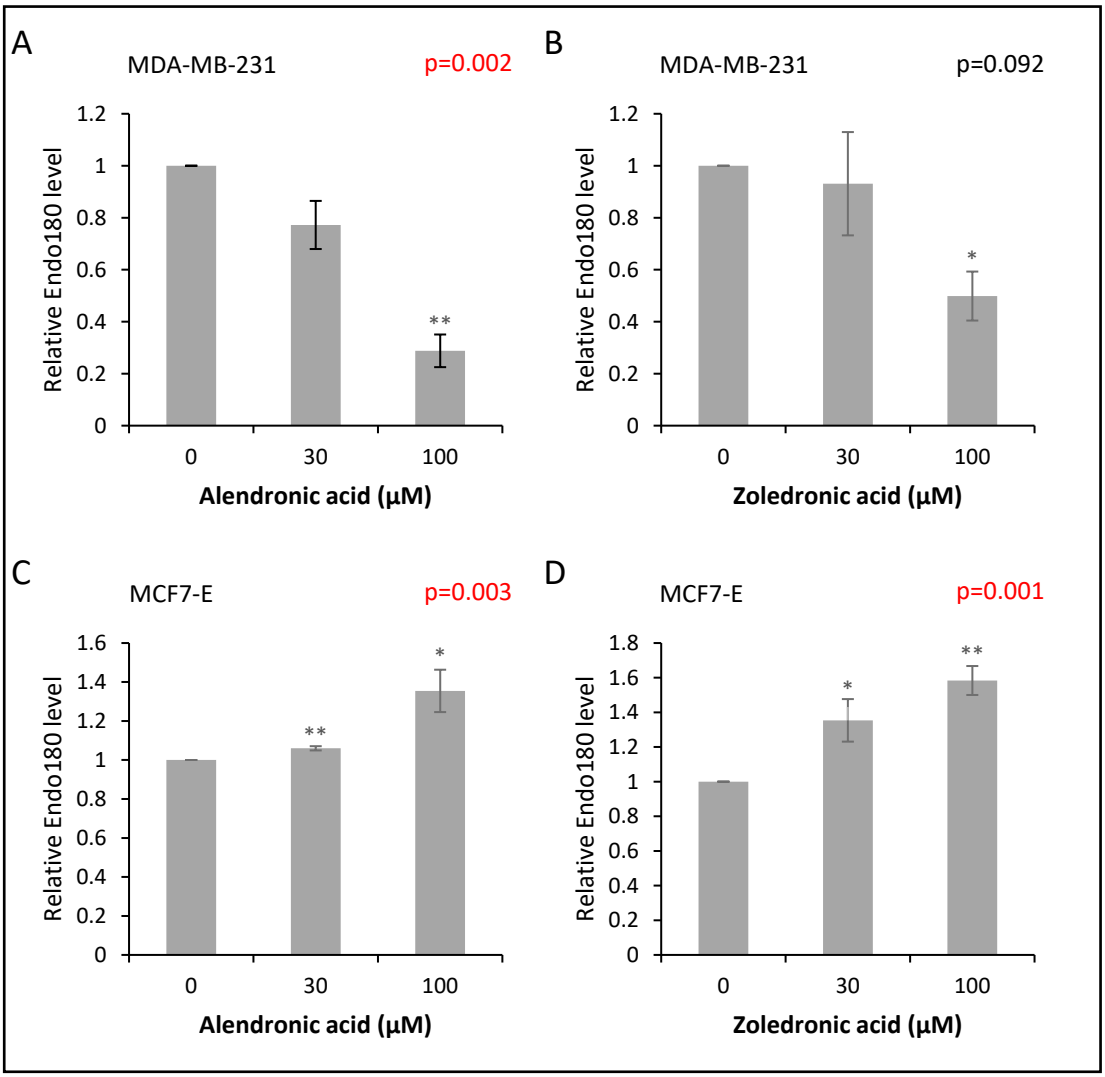
Based on the changes in the release of Endo180 from tumour cells and osteoblastic cells treated with bisphosphonates (Section 6.3.1), we further investigated whether bisphosphonates could influence the expression of Endo180 and the level of Endo180 on the cell surface, in order to understand the cause of the changes to the release of Endo180 under bisphosphonate treatment. Western blot was used to detect Endo180 in the lysate of treated cells and measure the total cellular expression levels of End180. In addition, flow cytometry was used to analyse the level of Endo180 on the surface of treated cells. Tumour cells and osteoblastic cells used in previous section were treated and analysed as described before.

As is shown in Figure 6.7, alendronic acid suppressed the expression of Endo180 in MDA-MB-231 in a dose-dependent manner, with a decrease in Endo180 expression of 22.8% – 71.2% ($P < 0.05$, t-test) at different treatment concentrations (Figure 6.7 A); zoledronic acid also suppressed Endo180 expression, however, the effect was not significant at lower treatment concentrations, with a small decrease of 6.9% at 30 μM ($P = 0.76$, t-test). At higher concentration, zoledronic acid showed an obvious inhibition on Endo180 expression by 50.1% ($P = 0.03$, t-test). Different from MDA-MB-231, the expression of Endo180 increased in MCF7-E under bisphosphonate treatment. Alendronic acid increased the expression of Endo180 in MCF7-E by 6.0% – 35.4% ($P < 0.05$, t-test, Figure 6.7 C), while zoledronic acid increased Endo180 expression by 35.4% – 58.4% ($P < 0.05$, t-test, Figure 6.7 D). The promotive effect on Endo180 expression by bisphosphonates in MCF7-E was dose-dependent.

As to osteoblastic cells, an up-regulation on Endo180 expression was observed with bisphosphonate treatment. In U-2 OS cells, the expression of Endo180 showed an increase of 24.2% – 26.4% ($P < 0.05$, t-test) by alendronic acid treatment (Figure 6.7

C), and showed an increase of 33.0% – 45.7% ($P < 0.05$, t-test) by zoledronic acid treatment (Figure 6.7 D). When treated with alendronic acid, osteoblasts showed no significant changes on Endo180 expression (Figure 6.7 E). In comparison to the down-regulation on Endo180 expression in MDA-MB-231 cells, the up-regulation by bisphosphonates in MCF7-E and U-2 OS was smaller and less marked.

Besides the cell lines mentioned above, we also explored the effect of bisphosphonates on Endo180 expression in MCF-7, PC-3, and RWPE-1 cells. The expression levels of Endo180 in MCF-7 and PC-3 were too low to be detected. In RWPE-1, there was no significant change in Endo180 expression under bisphosphonate treatment (Figure 6.8).



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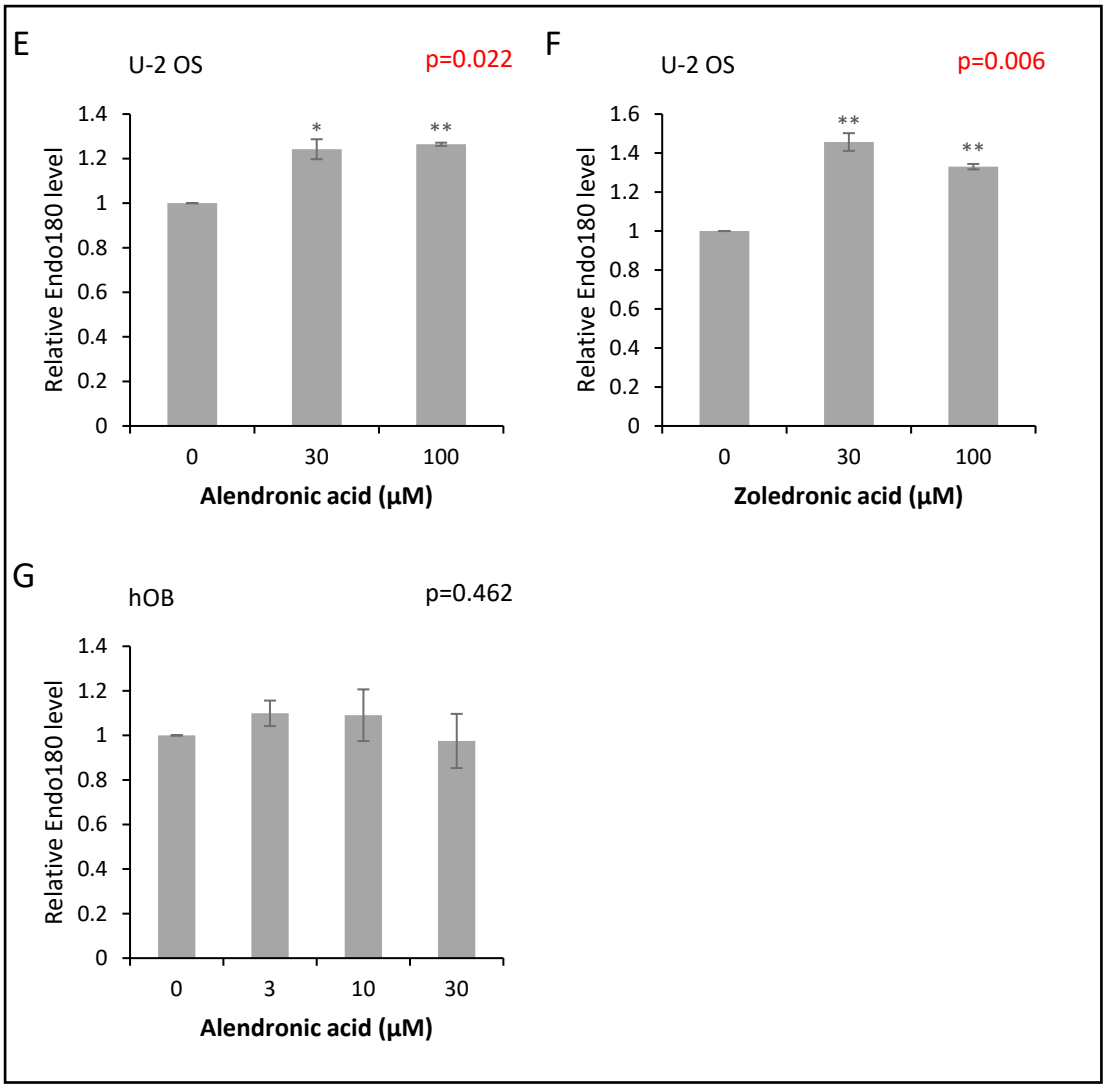
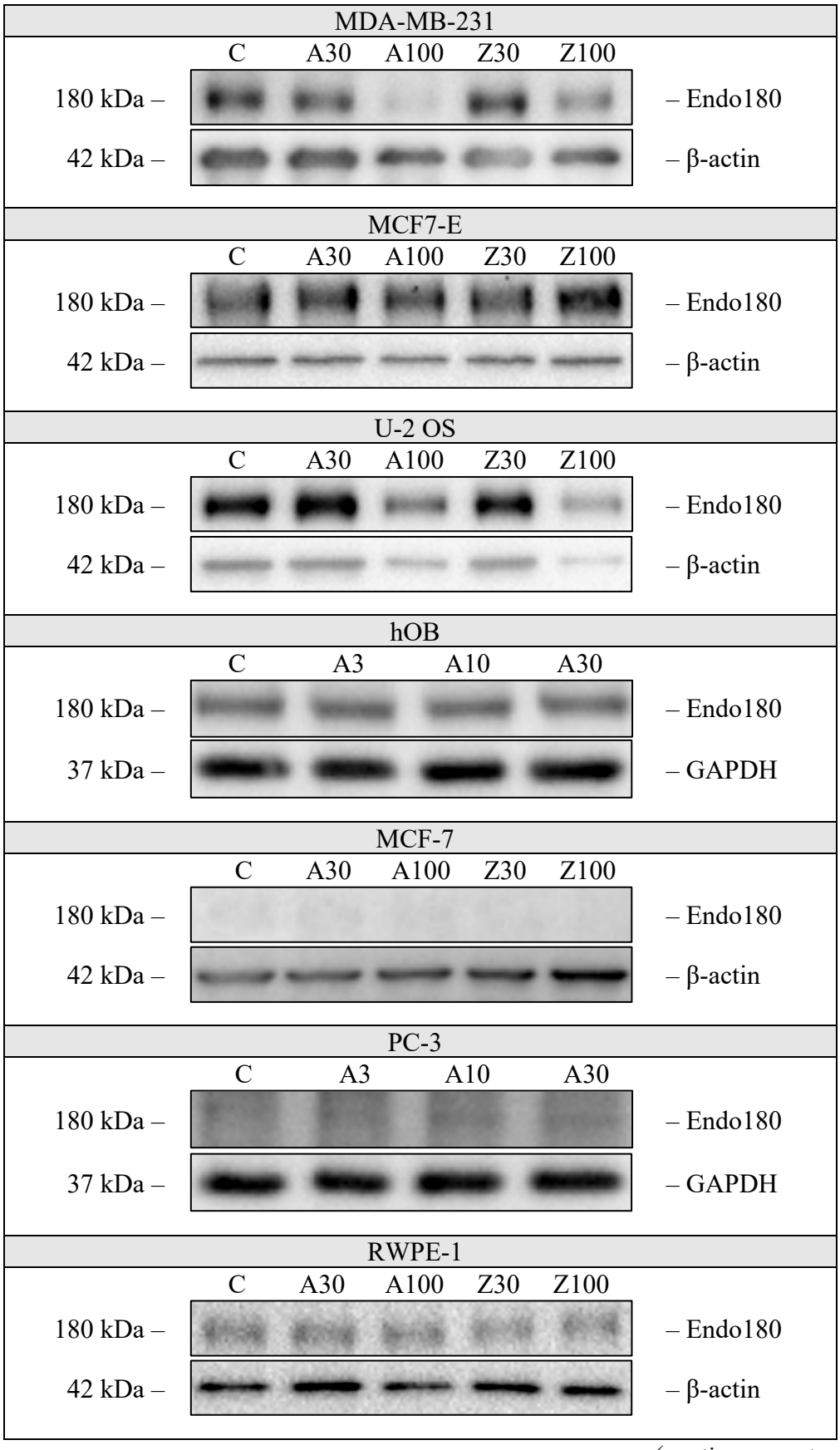


Figure 6.7 Western blot analysis of cells treated under bisphosphonate treatment

The level of Endo180 expression in MDA-MB-231 (A, B), MCF7-E (C, D), U-2 OS (E, F) and human osteoblasts (G) that were treated with 3 – 100 μM alendronic acid or zoledronic acid respectively for 48 hours was analysed using Western blot. The corresponding cell type with no treatment (0 μM) under the same growth condition were used as control. The relative Endo180 level was calculated by normalising the intensity of Endo180 bands to corresponding band of loading control (β-actin or GAPDH), and then comparing to the control (Endo180 level set as 1). Results were represented as mean ± SEM, n = 3, one-way ANOVA was used to test the significance of differences between treatments (where ANOVA is not applicable due to poor variance homogeneity, nonparametric Kruskal-Wallis test was used). $P \leqslant 0.05$ was considered as significant difference (marked in red). Student's t-test was used to compare the significance of the change in a specific treatment group with the control, * $P \leq 0.05$, ** $P \leq 0.01$.

A



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B

MDA-MB-231					
	C	A30	A100	Z30	Z100
Endo180	2983500	2285868	404872	2539160	1425970
β-Actin	6741592	6664008	5585346	4351854	4694352
MCF7-E					
	C	A30	A100	Z30	Z100
Endo180	5441580	4602096	5011038	5622966	6970266
β-Actin	1937655	1605285	1323567	1435185	1579149
U-2 OS					
	C	A30	A100	Z30	Z100
Endo180	4632600	5104525	2476025	4346875	1377125
β-Actin	2054050	1746425	870050	1340050	466500
hOB					
	C	A3	A10	A30	
Endo180	4457830	4252716	4001630	3931642	
GAPDH	3591562	3295240	3756194	3937414	
RWPE-1					
	C	A30	A100	Z30	Z100
Endo180	1956204	2274129	2200851	1857276	1903986
β-Actin	2513214	3143367	2446848	2696571	2350431

Figure 6.8 Representative Western blot data for Endo180 expression in cells treated with bisphosphonates

A) Human breast cancer cell lines MDA-MB-231 and MCF7-E, human osteoblast-like cell line U-2 OS, human prostate epithelial cell line RWPE-1 were treated with 30 μM (A30), 100 μM (A100) alendronic acid, or 30 μM (Z30), 100 μM (Z100) zoledronic acid respectively for 48 hours. Human prostate cancer cell line PC-3, human osteoblasts (hOB) were treated with 3 μM (A3), 10 μM (A10), 30 μM (A30) alendronic acid for 48 hours. Cells with no treatment under the same growth condition were used as control (C). After treatment, cells were collected, lysed, and was then analysed using Western blot. B) The density of the bands of Endo180 and the bands of loading control. The Endo180 bands of MCF-7 and PC-3 cells were too weak to be quantified.

With the treatment of bisphosphonates at high concentration (100 μM), the densitometry of the bands of loading control in U-2 OS cells is lower than other cell types This indicates that less cells were collected under treatments at high concentration, which might be a decrease in the number of cells caused by treatment. In order to investigate if bisphosphonates could lead to apoptosis of U-2 OS cells, TUNEL assay was applied to cells under the treatment of bisphosphonates. As is shown in Figure 6.9,

cells displayed no sign of apoptosis with or without the treatment of either alendronic acid or zoledronic acid at 30 µM and 100 µM (Figure 6.9).

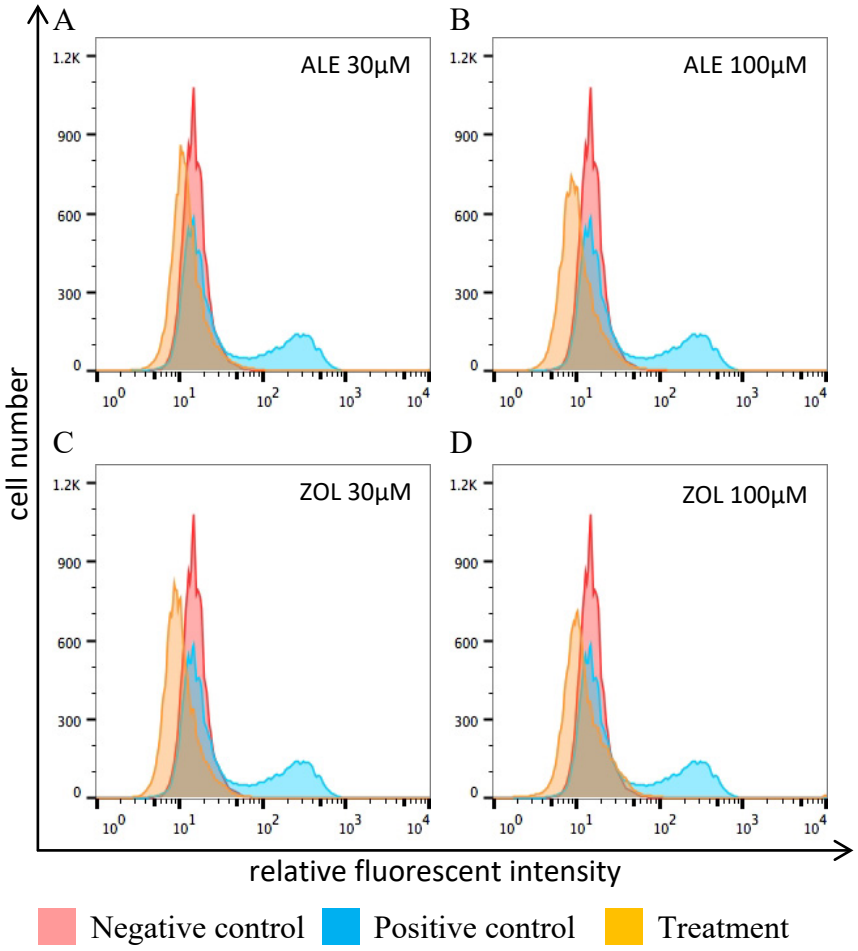


Figure 6.9 Detection of apoptosis in U-2 OS cells treated with bisphosphonates
U-2 OS cells were treated with 30 µM alendronic acid (A), 100 µM alendronic acid (B), 30 µM zoledronic acid (C), 100 µM zoledronic acid (D) for 48 hours respectively. Cells were collected, fixed, and stained to show the DNA break sites. Flow cytometry was used to analyse the fluorescent intensity of each group, which indicates the level of apoptosis under the corresponding treatment. ALE – alendronic acid, ZOL – zoledronic acid.

From the results in this section, we concluded that: 1) Endo180 expression in breast cancer cell MDA-MB-231 is inhibited by bisphosphonates in a dose-dependent manner; 2) bisphosphonates do not supress the expression of Endo180 in osteoblast and osteoblast-like cells; 3) Endo180 expression is upregulated by bisphosphonates in breast cancer cells that overexpress Endo180.

6.3.3 The level of Endo180 on the cell surface is decreased in tumour cells treated with bisphosphonates

As a cell membrane receptor which is constitutively recycling between cytoplasm and cell surface (Howard and Isacke, 2002), Endo180 could be released into the surrounding matrix directly from the cell membrane. Given that the release and expression of Endo180 is modulated by bisphosphonates, it was considered important to investigate if the level of Endo180 on the cell surface can be regulated by bisphosphonates, to obtain a comprehensive understanding of how Endo180 was regulated when different types of cells are treated with bisphosphonates. To achieve this goal, cells that received the same treatments as described in Section 6.3.1 and 6.3.2 were stained and analysed using flow cytometry, to measure the level of Endo180 on the cell surface. The relative Endo180 expression levels were calculated by normalising the geometric mean of the fluorescent intensity of the treatment groups to that of the control group. The statistical analysis of the results shows that:

Both alendronic acid and zoledronic acid showed an inhibitory effect on the level of Endo180 on the surface of MDA-MB-231 cells (Figure 6.10 A, B). The inhibition was dose-dependent, with a decrease of Endo180 level on cell surface by 9.7% – 31.1% ($P < 0.05$, t-test) under alendronic acid treatment, and 13.4% – 40.9% ($P < 0.01$, t-test) under zoledronic acid treatment. The changes between different treatment groups were also statistically significant ($P < 0.01$, one-way ANOVA). The effects of both drugs were more significant at high concentrations (30 – 100 μM) than low concentrations (3 – 10 μM);

The level of Endo180 on the surface of MCF7-E cells showed a decrease under alendronic acid treatment, and this inhibition was only statistically significant at high

treatment concentration (100 μM, p = 0.03, t-test). With the treatment of zoledronic acid, the level of Endo180 on cell surface showed a significant decreased of 26.9% – 40.5% ($P < 0.05$, t-test). The effect of both alendronic acid and zoledronic acid was dose-dependent, with significant differences between series concentrations within zoledronic acid treatment groups ($P = 0.003$, one-way ANOVA, Figure 6.10 C, D)

In U-2 OS cells, there was a significant decrease of 38.3% ($P = 0.001$, t-test) on cell surface Endo180 levels with alendronic acid treatment at high concentration (100 μM), while an increase of 11.9% ($P = 0.03$, t-test) was detected at low treatment concentration (30 μM). Similar effects were observed with zoledronic acid treatment, but was not statistically significant (Figure 6.11 A, B).

In osteoblasts, no significant change on the level of surface Endo180 was observed under the treatment of alendronic acid and zoledronic acid (Figure 6.11 C, D).

Besides the cell lines mentioned above, we also explored the effect of bisphosphonates on the level of cell surface Endo180 using MCF7-D, PC-3, and RWPE-1 cells. No significant change was detected to the level of cell surface Endo180 in MCF7-D cells under bisphosphonate treatment (Figure 6.12 A, B). PC-3 cells showed a similar pattern to breast cancer cells, with the level of cell surface Endo180 decreased by 11.7% – 44.8% under alendronic acid treatment, and 1.6% – 38.9% under zoledronic acid treatment. The effect of alendronic acid showed a dose-dependent manner ($P = 0.006$, one-way ANOVA), while 10 μM was the most effective treatment concentration under zoledronic acid treatment (Figure 6.12 C, D). In RWPE-1 cells, bisphosphonates also showed an inhibitory effect on the level of cell surface Endo180 (Figure 6.12 E, F).

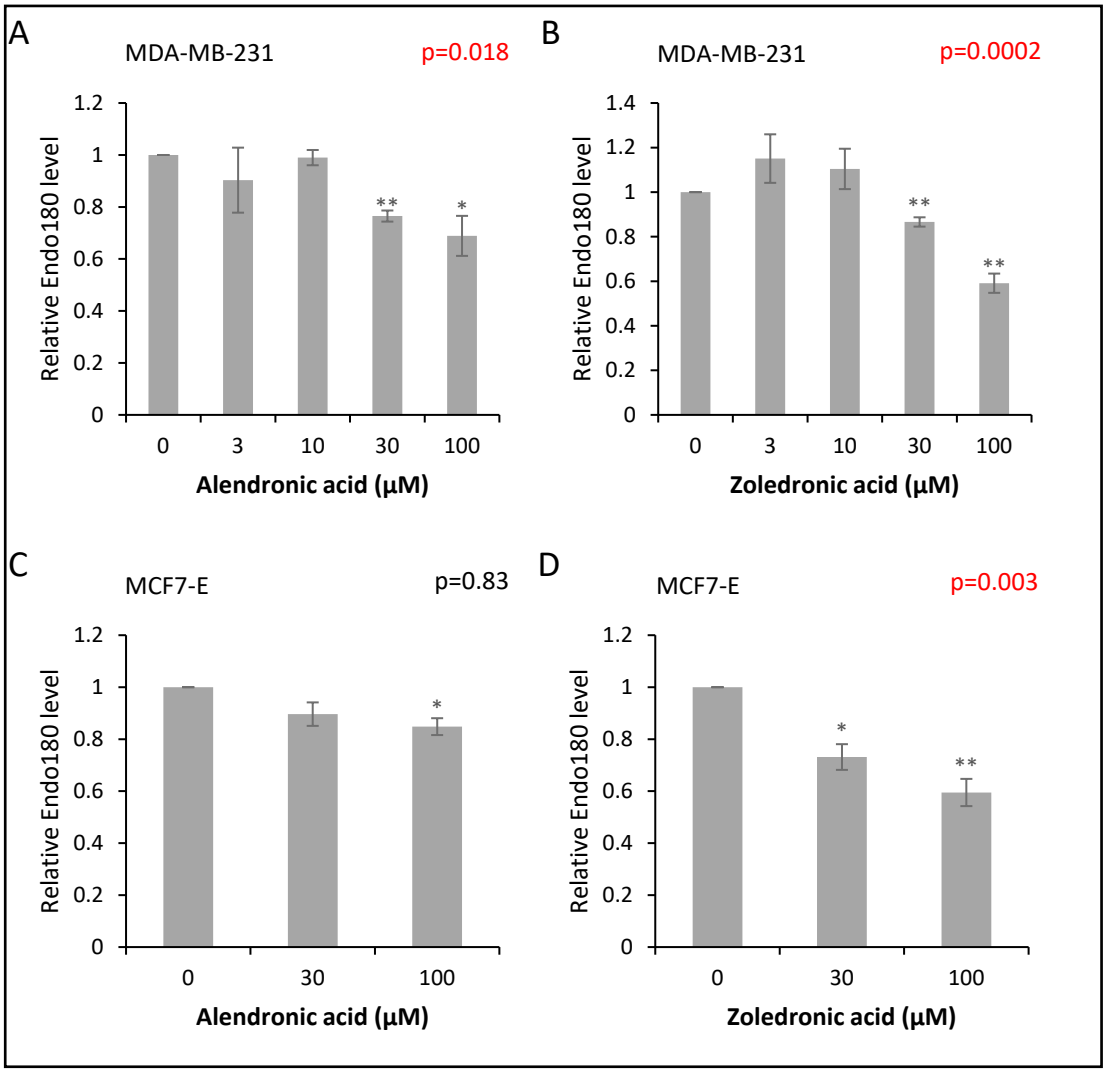


Figure 6.10 Flow cytometry analysis on cell surface Endo180 levels in MDA-MB-231 and MCF7-E cells treated with bisphosphonates

MDA-MB-231 (A, B) and MCF7-E (C, D) cells were treated with 3 – 100 μM alendronic acid or zoledronic acid for 48 hours. After treatment, cells were stained, fixed and analysed using flow cytometry to measure the level of Endo180 on the surface of cell. Relative Endo180 levels were calculated by normalising the fluorescent intensity of each treatment group to corresponding non-treatment control group (Endo180 level set as 1). Results were represented as mean ± SEM, n = 4 – 5, one-way ANOVA was used to test the significance of differences between treatments (where ANOVA is not applicable due to poor variance homogeneity, nonparametric Kruskal-Wallis test was used as a replacement). $P \leqslant 0.05$ was considered as significant difference. Student's t-test was used to compare the significance of the change in a specific treatment group with the control, * $P \leq 0.05$, ** $P \leq 0.01$.

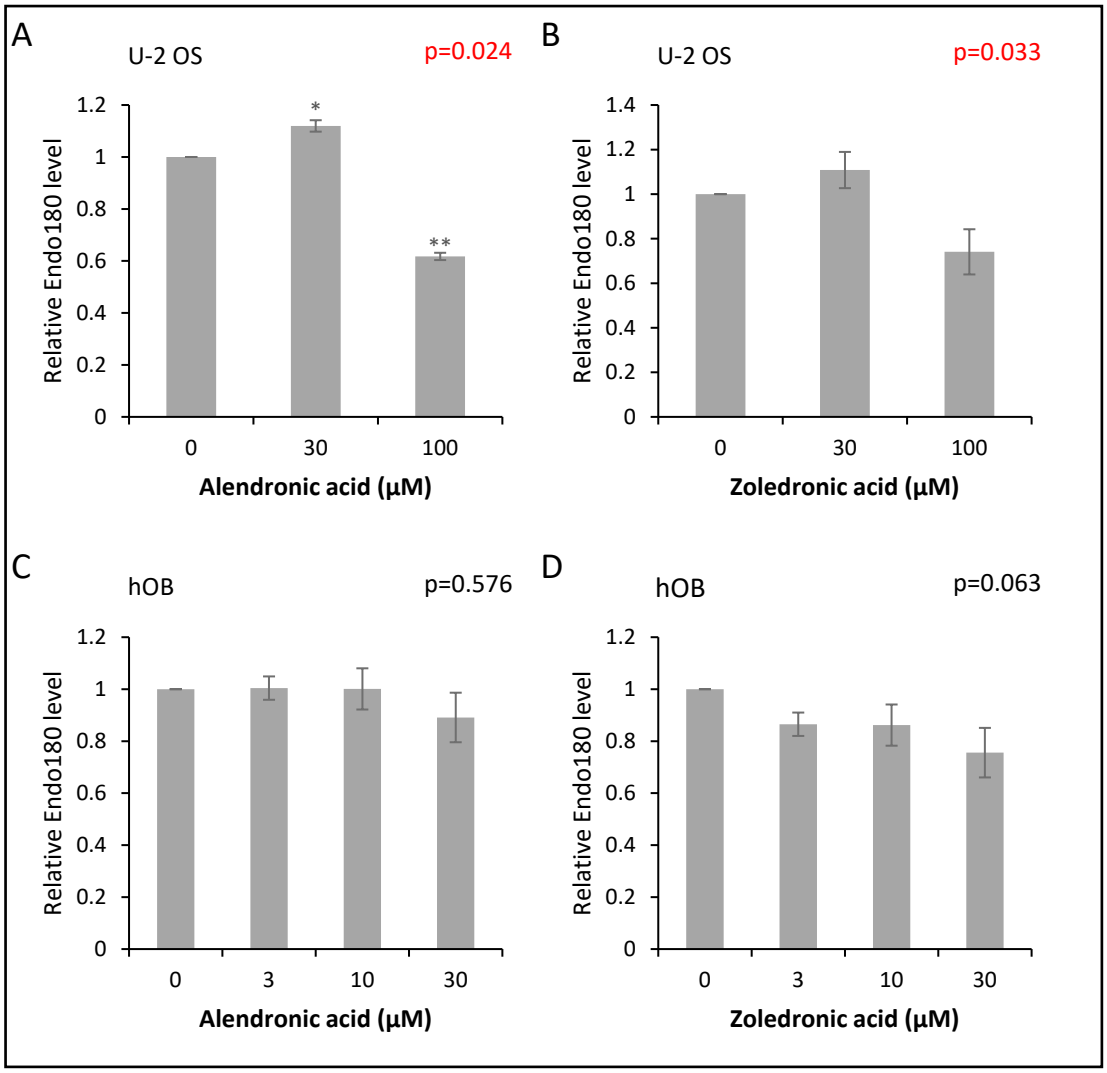


Figure 6.11 Flow cytometry analysis on cell surface Endo180 levels in U-2 OS and hOB cells treated with bisphosphonates

U-2 OS (A, B) and hOB (C, D) cells were treated with 3 – 100 µM alendronic acid or zoledronic acid for 48 hours. After treatment, cells were stained, fixed and analysed using flow cytometry to measure the level of Endo180 on the surface of cell. Relative Endo180 levels were calculated by normalising the fluorescent intensity of each treatment group to corresponding non-treatment control group (Endo180 level set as 1). Results were represented as mean ± SEM, n = 3, one-way ANOVA was used to test the significance of differences between treatments (where ANOVA is not applicable due to poor variance homogeneity, nonparametric Kruskal-Wallis test was used as a replacement). $P \leqslant 0.05$ was considered as significant difference. Student's t-test was used to compare the significance of the change in a specific treatment group with the control, * $P \leq 0.05$, ** $P \leq 0.01$.

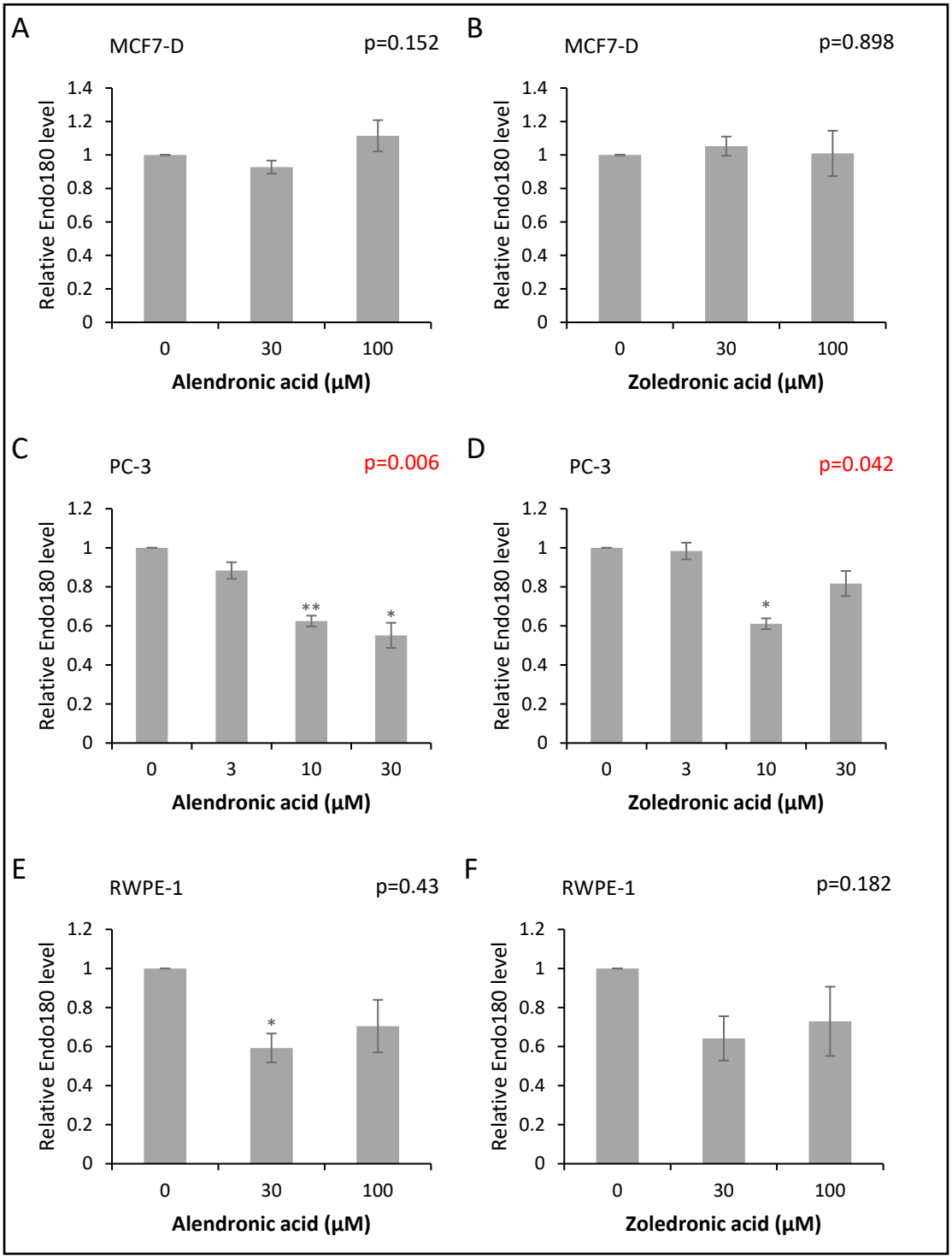


Figure 6.12 Flow cytometry analysis on cell surface Endo180 levels in MCF7-D, PC-3 and RWPE-1 cells treated with bisphosphonates

MCF7-D (A, B), PC-3 (C, D) and RWPE-1 (E, F) cells were treated with 3 – 100 μM alendronic acid or zoledronic acid for 48 hours. After treatment, cells were stained, fixed and analysed using flow cytometry to measure the level of Endo180 on the surface of cell. Relative Endo180 levels were calculated by normalising the fluorescent intensity of each treatment group to corresponding non-treatment control group (Endo180 level set as 1). Results were represented as mean ± SEM, n = 3 – 5, one-way ANOVA was used to test the significance of differences between treatments (where ANOVA is not applicable due to poor variance homogeneity, nonparametric Kruskal-Wallis test was used as a replacement). $P \leqslant 0.05$ was considered as significant difference. Student's t-test was used to compare the significance of the change in a specific treatment group with the control, * $P \leq 0.05$, ** $P \leq 0.01$.

6.4 Discussion

Table 6.1 Overview of the changes of Endo180 levels in cells treated with bisphosphonates

Alendronic acid			
	Release	Expression	Cell surface
MDA-MB-231	↓	↓	↓
MCF7-E	↑	↑	↓
U-2 OS	–	↑	↑
hOB	↑	–	–
Zoledronic acid			
	Release	Expression	Cell surface
MDA-MB-231	↓	↓	↓
MCF7-E	↑	↑	↓
U-2 OS	↑	↑	↑
hOB	↓	N/A	–

Note: this table provides an overview of Endo180 level changes in different formats in different cell lines. For each specific cell line, the comparison between the level of Endo180 in the treatment groups that were treated with 30 μM alendronic acid or zoledronic acid and the control groups with no treatment were used to generate the changing data. "↑" – an increase of Endo180 level was observed, "↓" – a decrease of Endo180 level was observed, "–" – no significant change in Endo180 level was observed.

In this chapter, we have investigated how two types of clinically used bisphosphonates, alendronic acid and zoledronic acid, modify the release, expression and cell surface level of Endo180 in tumour cells and osteoblasts. Bisphosphonates have been shown to be able to induce direct anti-tumour effects in breast cancer cells *in vitro*, and could also modify the osteoblastic niche *in vivo*, but it still remains to be investigated how bisphosphonates take effect in these biological contexts (Verdijk et al., 2007, Haider et al., 2014). It has also been suggested that bisphosphonates have the potential to modify the bone microenvironment surrounding the invaded tumour cells (van der Pluijm et al., 2005).

The key factor that we hypothesised to be regulated by bisphosphonates in the bone microenvironment with metastatic tumour cells is Endo180, which is revealed to be a potential biomarker for metastatic breast cancer (Chapter 4). There is evidence to support the role of Endo180 in the "vicious cycle" that occurs between the invasive tumour cells, osteoblasts and osteoclasts in the metastatic niche, which could

dysregulate the balance between bone resorption and production under normal circumstances, hence leads to a osteolytic or osteogenic lesion to bone (Caley et al., 2012). Together with the results presented in this chapter, this suggests that the lower plasma Endo180 levels observed in breast cancer patients treated with bisphosphonates is due to their ability to prevent Endo180 release (Palmieri et al., 2013a). A net effect of this blockade of Endo180 release by bisphosphonates could be the disruption of the "vicious cycle" leading to reduced bone resorption and increased bone production.

In order to get a comprehensive understanding of the effect of bisphosphonates on Endo180, *in vitro* experiments were set up to measure the level of Endo180 in the conditioned medium, the whole cell lysate, and on the surface of cells that were treated with bisphosphonates. These three types of sample represented the release, expression and cell surface level of Endo180 respectively. Given that bisphosphonates might affect the invasiveness of tumour cells and the bone-forming function of the stromal cells lining the bone surface in secondary bone tumours, we used both breast cancer cells and osteoblasts (osteoblast-like cells) in the study. We also used prostate cancer cells as an alternate tumour type that frequently metastasises to bone, and prostate epithelial cells as a model of glandular epithelium where Endo180 expression and its EMT suppressor function is required to maintain normal epithelial structure and function (Rodriguez-Teja et al., 2015b).

We first analysed the level of Endo180 release from the above cell lines (Figure 6.2). The results showed that osteoblastic cell lines (hOB and U-2 OS) release more Endo180 than breast and prostate cancer cells. Among tumour cells, cells with mesenchymal phenotype (MDA-MB-231) release more Endo180 than cells with epithelial-like phenotype (MCF-7 and PC-3). This is consistent with the expression level of Endo180 in different cell lines, which is correlated with the phenotype and collagen

internalisation capability of the cell (Madsen et al., 2011). Moreover, when the breast cancer cell line MCF-7 is transfected to overexpress wild-type Endo180 (MCF7-E), the level of Endo180 release was increased accordingly. MCF7-E cells were shown to obtain the collagen uptake capability and become more mesenchymal in previous studies (Sturge et al., 2003, Sturge et al., 2006, Wienke et al., 2007). These results indicate that: 1) the release of Endo180 is mainly determined by the expression level of Endo180; 2) similar to Endo180 expression, the release of Endo180 might also correlate to and reflect the mesenchymal phenotype and the capability in collagen uptake of different cell types.

In breast and prostate cancer cells, the level of Endo180 expression showed a decrease under the treatment of bisphosphonates, which means that bisphosphonates could inhibit the expression of Endo180 in tumour cells (Table 6.1). As Endo180 plays an important role in collagen uptake, bone degradation and cell migration, the down regulation of Endo180 by bisphosphonates suggests that the inhibition of Endo180 could be part of the anti-tumour effects of bisphosphonates (Madsen et al., 2011, Caley et al., 2016, Engelholm et al., 2016). In comparison to the decrease of the expression and release (22.8% – 71.2%), the down- regulation of the cell surface Endo180 level (9.7% – 31.1%) is relatively lower. As an endocytic recycling transmembrane glycoprotein, Endo180 is constitutively recycling between cell surface and endosome, to carry out the role in collagen uptake and turnover (Isacke et al., 1990, Sheikh et al., 2000). The decreased level of Endo180 down-regulation on cell surface suggests tumour cells might have a resistant response to the inhibition of Endo180 expression by bisphosphonates. Although the overall expression level of Endo180 decreases when treated with bisphosphonates, tumour cells could still use existing Endo180 which has already been produced, and increase the speed of Endo180 recycling, in order to

maintain levels of Endo180 on cell surface, and the essential functions of Endo180 in collagen turnover and bone degradation, and the invasive tumour phenotype.

The decrease in the level of Endo180 release into cell growth medium under the treatment of bisphosphonates is supposed to be a collective effect of reducing Endo180 expression and the resistant response to bisphosphonates on maintaining Endo180 recycling in tumour cells. As the cells use the limited amount of the existing Endo180 to maintain the recycling between cell surface and endosome, the release of Endo180 would decrease due to the decrease of Endo180 expression.

As an important component of the bone microenvironment, osteoblasts is shown to be part of the "vicious cycle" induced by the invasive tumour cells to the metastatic niche (Käkönen and Mundy, 2003). A recent study has shown that bisphosphonates could modify the osteoblastic niche by inducing a decrease in osteoblastic cells lining on bone surface (Haider et al., 2014). Given that Endo180 participates in the collagen deposition by primary human osteoblasts, it was considered necessary to explore if the expression and release of Endo180 in osteoblasts is also modulated by bisphosphonates (Caley et al., 2012). The results of the experiments presented in this chapter show that bisphosphonates do not inhibit the expression of Endo180 in osteoblastic cells, on the contrary, there was an increase of Endo180 in hOB and U-2 OS cells under the treatment of bisphosphonates. As to the level of Endo180 released from osteoblastic cells, no statistically significant effect was observed in cells treated with bisphosphonates ($P > 0.05$ in t-test of all treatment groups). The level of Endo180 on the surface of osteoblasts and U-2 OS showed a decrease with bisphosphonate treatment, and the effect was more significant in U-2 OS cells. The unaffected Endo180 expression and surface level indicates that it is unlikely that Endo180-dependent osteoblastic functions are affected by bisphosphonates.

MCF7-E cells showed an upregulation on Endo180 expression and release levels under bisphosphonate treatment, while the cell surface Endo180 level decreased. As a stably transfected breast cancer cell line that overexpresses Endo180, the expression and release of Endo180 in MCF7-E cells are always at a higher level than non-transfected cells, and might not be controlled by the normal regulation pathway as in non-transfected breast cancer cells. Interestingly, the regulation of Endo180 by bisphosphonates in MCF7-E cells is more like the effect of bisphosphonates in osteoblastic cells. This indicates that the expression and release of Endo180 in tumour cells and osteoblasts are not only at a different level, but also can be regulated by different pathways. It would be useful to investigate if there are more than one regulatory pathways for the expression and release of Endo180 exist, and if Endo180 plays different biological roles in tumour cells and osteoblasts, in order to help understand the relationship between Endo180 expression and its function in different cell types. Recent studies found that zoledronic acid could only inhibit the proliferation of ER negative breast cancer cells MDA-MB-231 and MDA-MB-436, but not ER positive MCF-7, T47D cells (Wilson et al., 2015). This could be another reason underlying the opposite effect of bisphosphonates on Endo180 expression and release in MCF7-E cells compared to MDA-MB-231 cells.

To fully understand the mechanism(s) underlying the differential regulatory effects of bisphosphonates on Endo180 expression and release, further investigations are required. The major limitations of the experiments performed in this study is the lack of control experiments to validate that the effect on Endo180 release and expression under bisphosphonates treatment is the direct effect of bisphosphonates, instead of the side effect resulted from the inhibition of cell proliferation or the introduction of apoptosis. To validate this, it is necessary to reverse one of the signalling pathways that are known

to be inhibited by bisphosphonates, such as the mevalonate pathway, and investigate whether this could rescue the inhibition of Endo180 release / expression caused by bisphosphonates. Furthermore, considering the relatively short half-life in circulation of bisphosphonates, and the uneven distribution throughout the body, it is necessary to investigate the effect of bisphosphonates under a wider concentration and duration range (Cremers and Papapoulos, 2011).

7 The investigation of the mechanisms regulating the release of Endo180

7.1 Overview

In previous chapters, we have demonstrated that the level of plasma Endo180 showed a significant difference between early and advanced breast cancer patients. We also showed that Endo180 is released from breast cancer cells and osteoblasts, and the release of Endo180 is modulated by bisphosphonate treatment. However, little is known about the mechanism of the release of Endo180 from cells, including the molecular mediators / regulators Endo180 release, and conditions or environment that can trigger the release of Endo180. The aim of this chapter was to explore the mechanism of Endo180 release, so as to understand the reasons for the change in the level of plasma Endo180 in breast cancer patients.

The first hypothesis was that MMPs could modulate the release of Endo180 from breast cancer cells. MT1-MMP have been shown to cooperate with Endo180 during collagen internalisation and degradation, and a potential MMP-2 cleavage site in the transmembrane domain of Endo180 has been predicted using bioinformatic tools (Madsen et al., 2007, Wagenaar-Miller et al., 2007, Kogianni et al., 2009). A recent study also showed that Endo180 could form a complex with CD147, a type I integral membrane receptor with the function of intercellular recognition, and the Endo180-CD147 complex is proved to be an EMT suppressor, the stability of which is important in maintaining the cell-cell junctions and the acini formed by prostate epithelial cells (Rodriguez-Teja et al., 2015b). Based on these findings, we hypothesised that MMP-2 and its regulator MT1-MMP might be functional in the disruption of Endo180-CD147 complex and the shedding of Endo180 from the surface of breast cancer cells. As bisphosphonates showed an inhibition on MMP-2 in human osteoblasts, it was

supposed that the same effect of bisphosphonates might also exist in breast cancer cells (Ichinose et al., 2000).

The second hypothesis was that the indirect coculture of breast cancer cells and osteoblasts affects the release of Endo180 from both cell types. In the advanced stage of breast cancer, tumour cells have migrated from the primary site and metastasised to remote sites, such as bone, lung, or other organs. As the level of plasma Endo180 is higher in advanced breast cancer patients, we supposed that the invasion of tumour cells to remote sites might modulate the level of Endo180 released by breast cancer cells and osteoblasts.

Our result showed that: (a) Endo180 expression could stimulate MMP-2 / -9 activity in MCF-7 cells; (2) MMP-2 could not directly cleave Endo180 from cell surface, but breast cancer cells treated with MMP-2 showed an increased level of un-complexed Endo180 on the cell surface, while the total level of Endo180 did not change; (3) MMP inhibitor could inhibit the release of Endo180, and increase the level of Endo180 on the surface of breast cancer cells; (4) Indirect contact between tumour cells and osteoblasts works differently in breast cancer and prostate cancer. In breast cancer, the co-culture does not affect the release of Endo180 from breast cancer cells, and supress the release of Endo180 from osteoblasts. In prostate cancer, the co-culture triggers the intake of Endo180 into prostate cancer cells, and increases the cell surface Endo180 level in osteoblasts.

7.2 Experimental setup

To verify if MMP-2 itself, or together with other MMPs, participates in the cleavage of Endo180 from cell surface, and to investigate if bisphosphonates affect the activities of MMP-2/-9, MDA-MB-231, MCF7-E, MCF-7, PC-3 and hOB cells were recovered and cultured as described in Section 3.2.1. Cells were detached and seeded into 6-well plates

at a concentration of 2×10^5 cells/well once they were sub-confluent in T75 tissue culture flasks. Cells were allowed to adhere and expand for 24 hours before being starved in serum-free medium for another 24 hours. For bisphosphonates treatment on MCF-7 and MCF7-E cells, the conditioned media containing different concentrations of alendronic acid or zoledronic acid was added to the treatment groups. For MMP-2 treatment on MDA-MB-231 and MCF7-E cells, the conditioned media containing different concentrations of MMP-2 was added to the treatment groups. For MMP inhibitor treatment on MDA-MB-231 and MCF7-E cells, the conditioned media containing different concentrations of Marimastat was added to the treatment groups. Control groups were treated with same volumes of corresponding conditioned medium without bisphosphonates. After 48-hour treatment, the medium was collected, centrifuged at 12,000 rpm, 4℃ for 10 minutes. The level of Endo180 in the collected conditioned medium was measured using the Endo180 ELISA developed in this study as described in Section 3.2.6.2. To measure the level of Endo180 expression, the cell monolayer was trypsinaised, collected, and the centrifuged at 4℃, 1,000 rpm for 3 minutes, lysed as described in Section 3.2.3, and then analysed using Western blot as described in Section 3.2.8. To measure the level of Endo180 on cell surface, live cells were harvested and analysed using flow cytometry as described in Section 3.2.9.

To investigate if the expression of Endo180 affects MMP-2 / -9 activities, gelatin zymography was used to compare the MMP-2 / -9 activities in conditioned media collected from MCF-7 and MCF7-E cells. The cells were also treated with bisphosphonates, to determine if MMP-2 / -9 activities are regulated by bisphosphonates, and if this regulation correlates with the changes of Endo180 levels by bisphosphonate treatment (Figure 7.1).

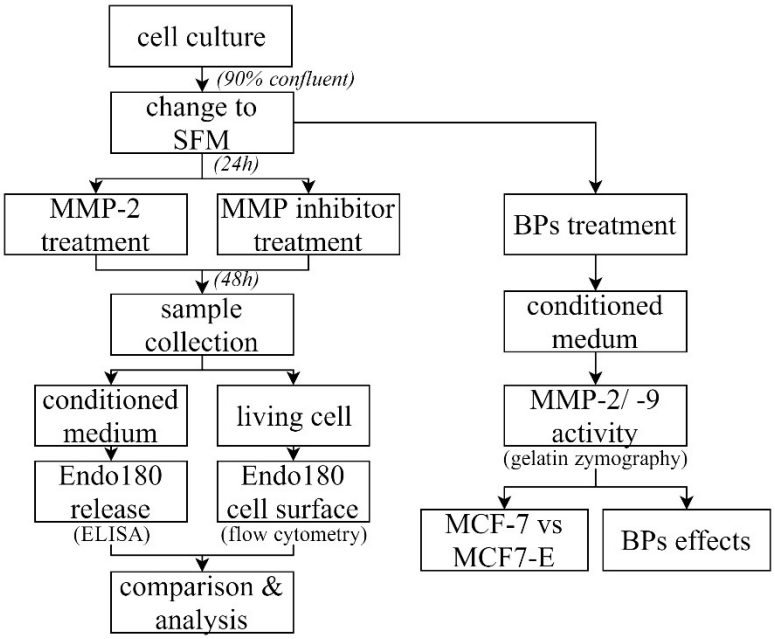


Figure 7.1 Design of MMP-2 & MMP inhibitor treatment experiments
Note: SFM – serum free medium, BPs – bisphosphonates (alendronic acid or zoledronic acid).

To investigate if the contact between tumour cells and osteoblasts could influence Endo180 release from both cell types, a co-culture experiment was set up using human breast cancer cells line MDA-MB-231, human prostate cancer cell line PC-3, and human osteoblast cells. The co-culture experiment was designed as an indirect co-culture, so that the changes in Endo180 released from tumour cells and osteoblasts can be measured distinguished. Breast cancer / prostate cancer cells and osteoblasts were cultured under normal growth conditions. Conditioned medium from one cell line was collected and used in the co-culture experiment on the other cell line. Detailed co-cultured procedure is described in Section 3.2.2. The levels of Endo180 in the conditioned medium from tumour cells and osteoblasts were measured using Endo180 ELISA before and after the 48-hour co-culture, to determine the changes in the release of Endo180 from each cell type. The changes were compared with the Endo180 release of control cells under normal growth conditions (Figure 7.2).

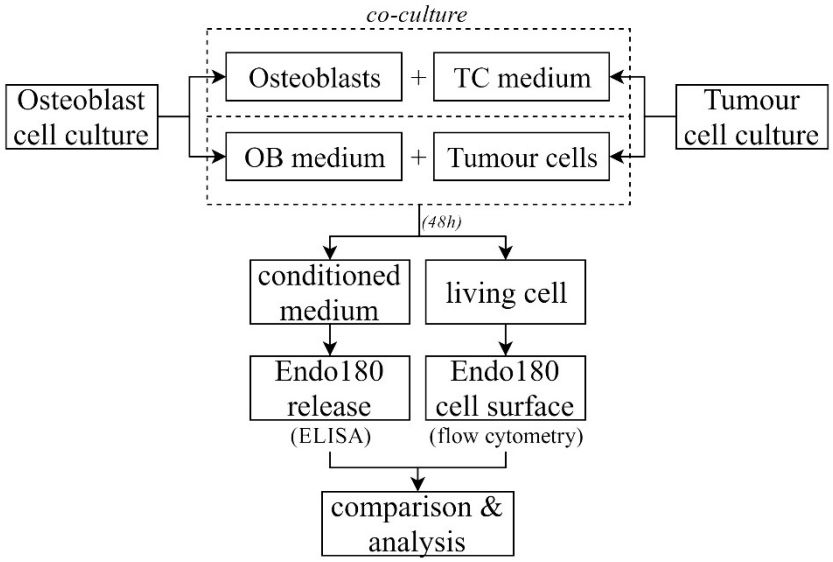


Figure 7.2 Design fo indirect co-culture experiments
Note: OB – osteoblast, TC – tumour cell.

7.3 Results

7.3.1 The interaction between Endo180 and MMPs

A cofunction between MT1-MMP and Endo180 has been revealed in the procedures of collagen remodelling and tumour cell migration (Madsen et al., 2007, Wagenaar-Miller et al., 2007, Kogianni et al., 2009). The up-regulation of MMP-2 expression and activity in breast cancer cell line MDA-MB-231 might contribute to the enhanced invasive potential of the cells (Baum et al., 2007), Bisphosphonates are shown to be able to inhibit and down-regulate the expression of MMPs (Teronen et al., 1999). In combination with the findings in this study that bisphosphonates could regulate the release and expression of Endo180, we hypothesized there might be an interaction between Endo180 and MMPs, which could be important in tumour migration, and could be affected by bisphosphonates.

7.3.1.1 The Effect of MMPs on the release of Endo180

We first investigated whether MMPs could mediate the release of Endo180 in breast cancer cells. Because the computational model has predicted a cleavage site of MMP-2 on the trans-membrane region of Endo180, we chose MMP-2 as an object of study,

to explore if it could affect the release of Endo180 from cell surface. MDA-MB-231 and MCF7-E cells were treated with active recombinant MMP-2. The level of Endo180 in the conditioned medium from treated cells and control cells were compared. Analysis using the Endo180 ELISA showed that an exposure of breast tumour cells to MMP-2 at the concentration up to 400 ng/mL did not show significant effect on the release of Endo180 from the cell surface (Figure 7.3). Based on this result, we concluded that MMP-2 does not directly mediate the cleavage of Endo180 from the surface of breast cancer cells.

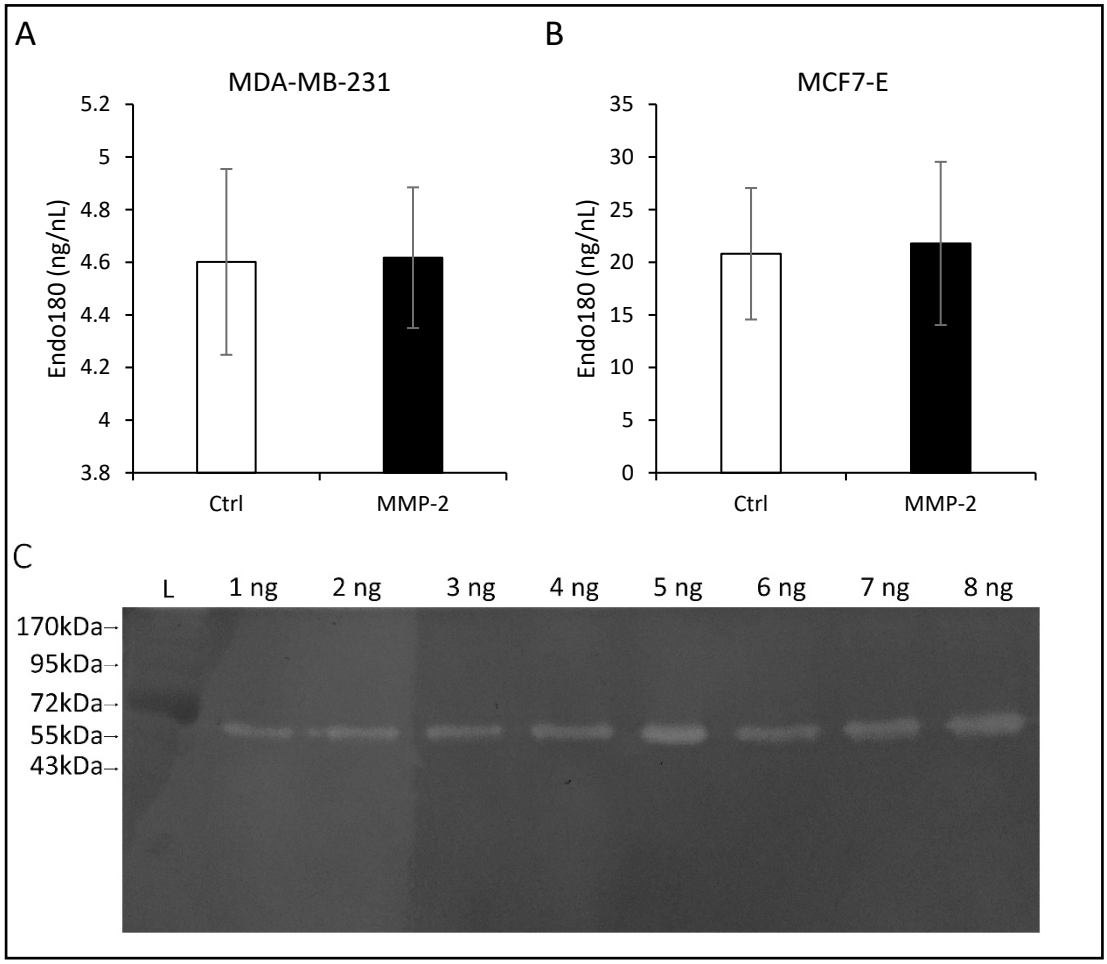


Figure 7.3 The effect of MMP-2 on the release of Endo180 from MDA-MB-231 and MCF7-E cells

Breast cancer cell lines MDA-MB-231 (A) and MCF7-E (B) were treated with 100 ng/mL MMP-2 for 48 hours. Conditioned media was collected and analysed using Endo180 ELISA to measure the level of Endo180. Results were represented as mean ± SEM, n = 3 – 4, student's t-test was used to compare the difference between treatment group and control group, no significant was observed in both treatment groups. (C) the activity of the recombinant human MMP-2 protein used in the treatment experiments was verified using gelatin zymography, 1 – 8 ng MMP-2 was added to each well. Clear and specific bands of MMP-2 (63 kDa) were detected. L – protein ladder.

We also measured the level of Endo180 on the surface of MDA-MB-231 cells under active MMP-2 treatment, to further confirm the functions of MMP-2 in the cleavage of Endo180 from cell surface. Similar to the level of Endo180 in the conditioned medium, there was no significant change in the total amount of Endo180 on cell surface. However, MMP-2 appeared to be able to increase the amount of single molecular Endo180 protein. When Endo180 forms complex with other molecules like uPA/uPAR or CD147, the epitope of mAb 39.10 on Endo180 will be blocked (Behrendt et al., 2000, Rodriguez-Teja et al., 2015b). Therefore, measurement of 39.10-stained cell using flow cytometry could reveal the level of single molecular Endo180 on cell surface, while the A5/158 staining reflects the total Endo180 level on cell surface. As is shown in Figure 7.4, there was an increase (1.5-fold, $P \leq 0.01$) on the level of single molecular Endo180 on cell surface with MMP-2 treatment, while the level of total Endo180 did not change. In combination with the results of the unaffected Endo180 release level, the results indicate that although MMP-2 dose not mediate the cleavage of Endo180 from cell surface, it could disrupt the complex formed between Endo180 and other molecules, inhibiting the complex-dependent cell functions. This could also be a precondition for the cleavage of Endo180 by other factors.

To confirm if other types of MMPs are functional in the procedures of Endo180 release, we used a broad-spectrum MMP inhibitor, Marimastat, to investigate if the inhibition of MMPs could influence the release of Endo180 from breast cancer cells. MDA-MB-231 and MCF-7 were treated with Marimastat at a series of concentrations between 2.5 – 10 μM for 48 hours, the level of Endo180 in the conditioned media was analysed by Endo180 ELISA and compared with control. The results showed that MMP inhibitor treatment markedly reduced the Endo180 levels in the conditioned media from both MDA-MB-231 and MCF-7 cells, with an inhibition of 64% – 67% in MDA-MB-231

(P < 0.05, t-test of each treatment), and 23% – 30% in MCF7-E (P < 0.05, t-test of each treatment). The dose-dependent effect of MMP inhibitor treatment was not significant, as the level of inhibition was similar between different treatment concentrations (Figure 7.5).

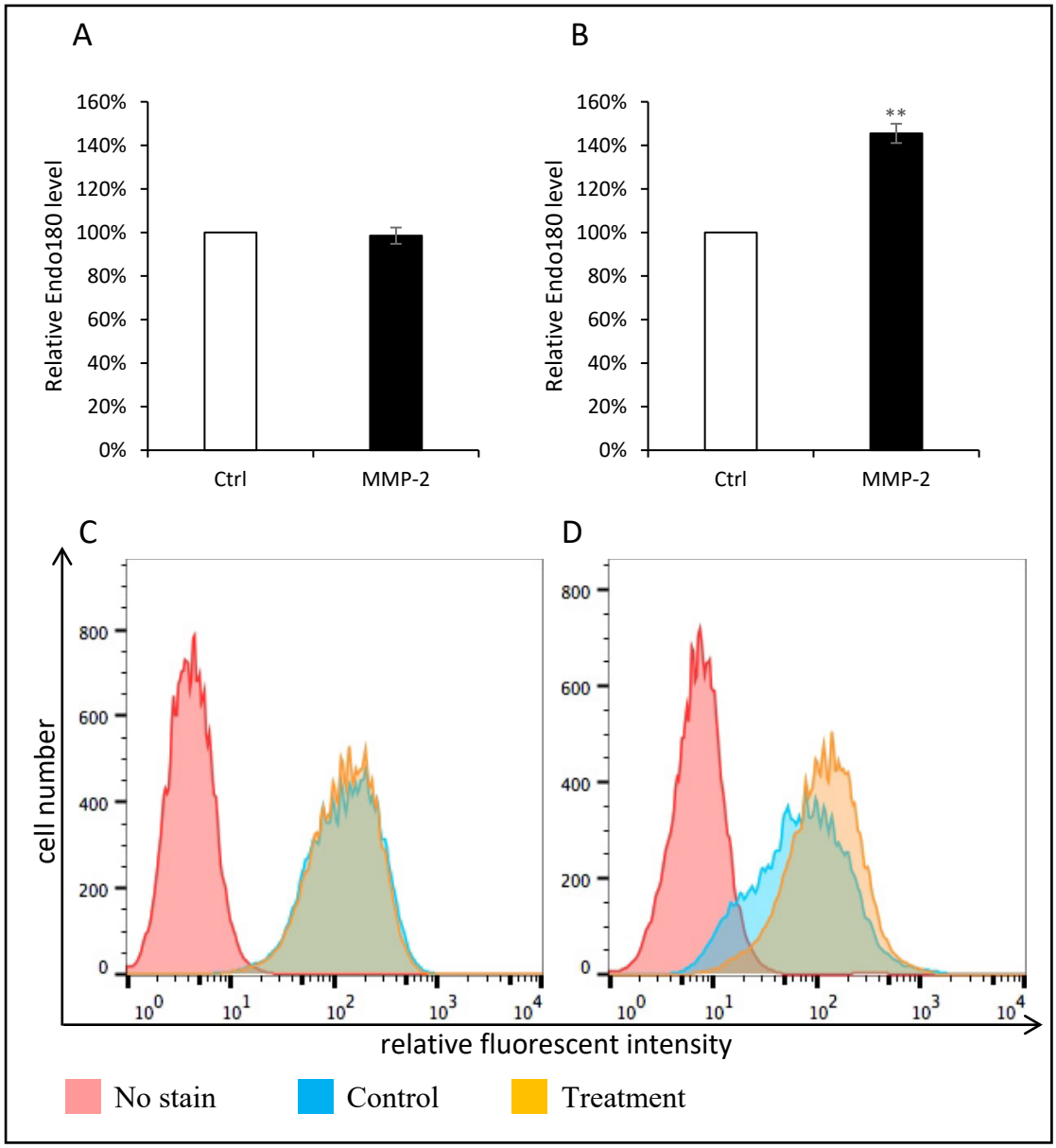


Figure 7.4 The effect of MMP-2 on cell surface Endo180 levels

Breast cancer cell line MDA-MB-231 were treated with MMP-2 for 48 hours. Cells were stained and fixed before being analysed using flow cytometry. Two primary antibodies were used to stain Endo180 at different state: A5/158 stains all Endo180 on cell surface (A; C), and 39.10 stains Endo180 in single molecular form on cell surface (B; D). Relative Endo180 level was calculated by normalising the fluorescent intensity of each treatment group to that of the control cells (Endo180 level set as 100%). (A, B): Statistical analysis of results was represented as mean ± SEM, n = 4 – 6. t-test was used to compare the difference between treatment group and control group, ** P < 0.01. (C, D) representative histograms of flow cytometry analysis using two antibodies with MMP-2 treated cells.

The level of Endo180 on cell surface was also affected by MMP inhibitor. Flow cytometry showed that there was a significant increase in Endo180 level on the surface of breast cancer cells with MMP inhibitor treatment (Figure 7.6). The amount of total Endo180 and single molecular Endo180 were both increased, and the increase of single molecular (70% – 93%) was higher than the total amount (19% – 41%).

The results of MMP-2 treatment and MMP inhibitor treatment indicate that MMP-2 could break the complex formed by Endo180 and other molecules, but does not directly mediate the release of Endo180 from the surface of tumour cells, other MMPs might work together with MMP-2 in the Endo180 shedding procedure.

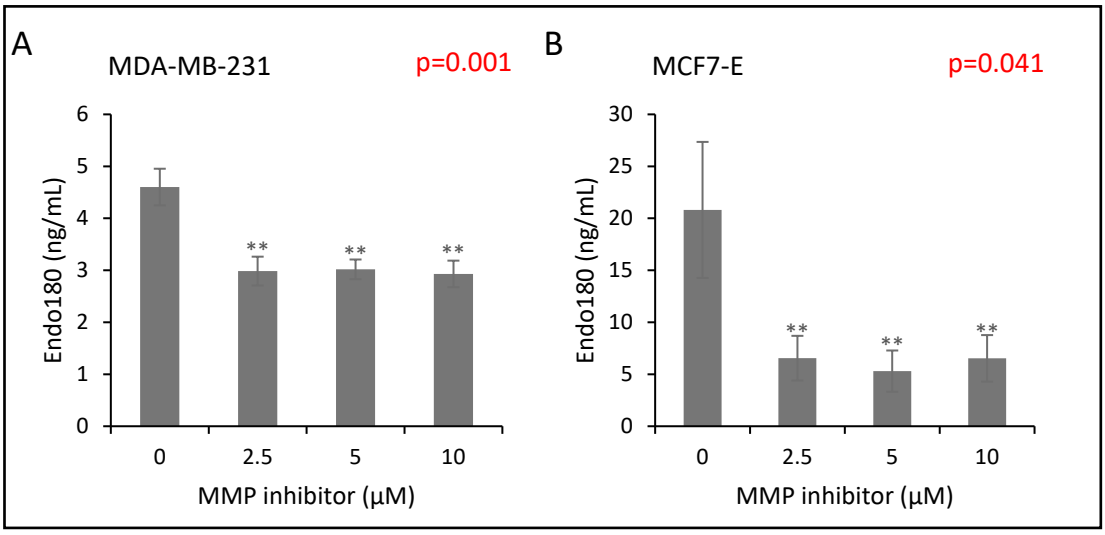


Figure 7.5 The effect of MMP inhibitor on the release of Endo180 from tumour cells

Breast cancer cells were treated with serial concentrations (2.5 - 10 μM) of MMP inhibitor for 48 hours. Conditioned media were collected and analysed using Endo180 ELISA to measure the level of Endo180. (A) MDA-MB-231; (B) MCF7-E. Results were represented as mean ± SEM, n = 3 – 4, one-way ANOVA was used to test the significance of differences between treatments in MDA-MB-231, and nonparametric Kruskal-Wallis test was used in MCF7-E. $P \leqslant 0.05$ was considered as significant difference (marked red). Student's t-test was used to compare the significance of the change in a specific treatment group with the control, * $P \leq 0.05$, ** $P \leq 0.01$.

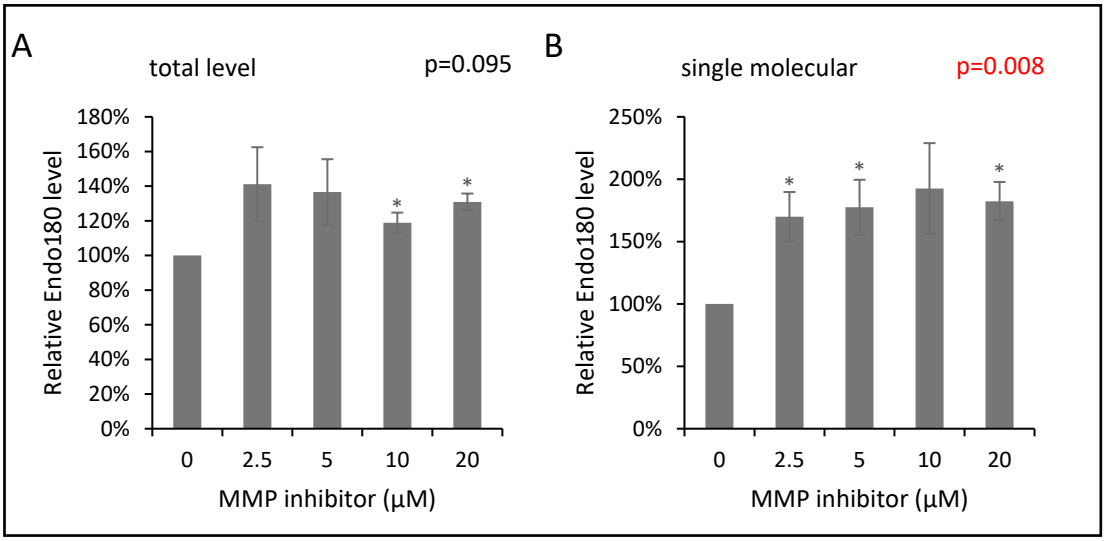
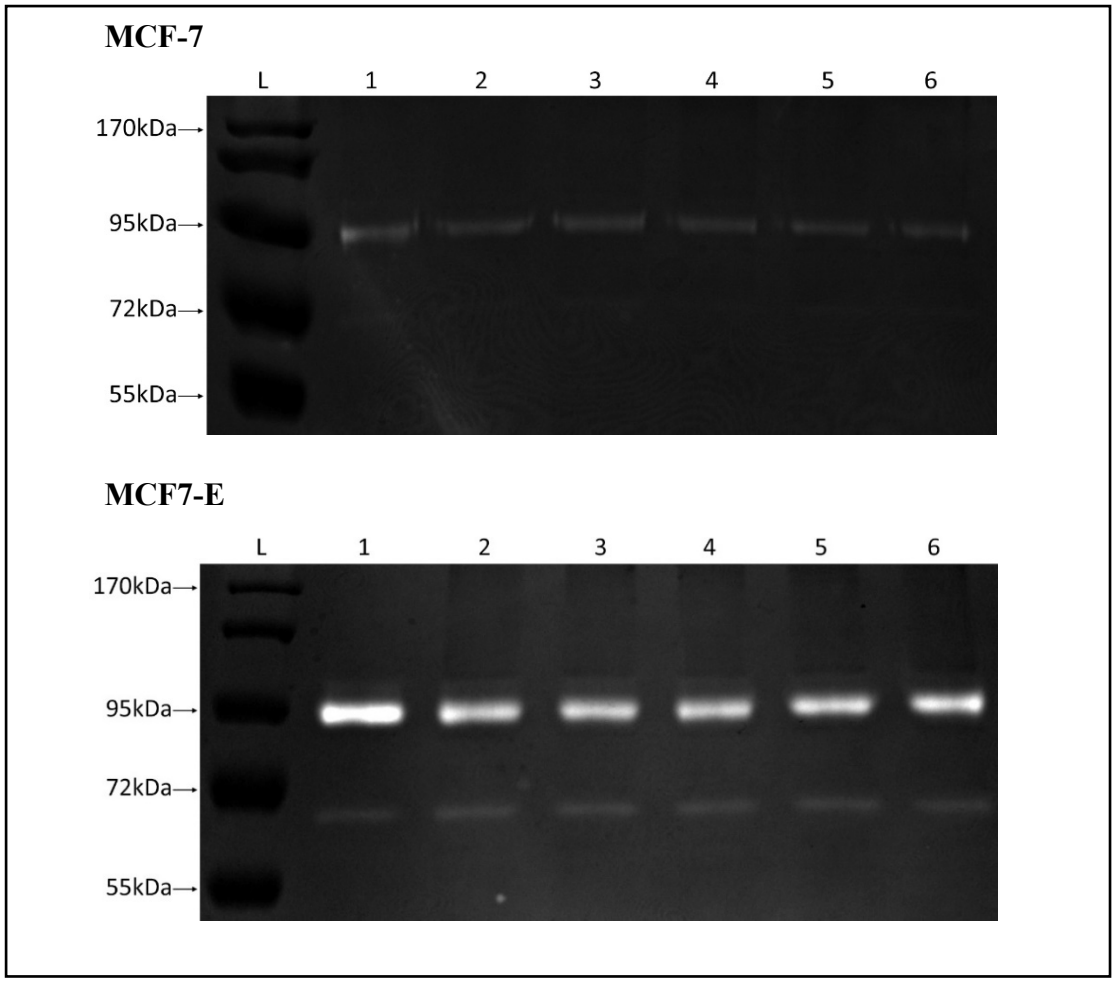


Figure 7.6 The effect of MMP inhibitor on cell surface Endo180 levels

MDA-MB-231 cells were treated with series concentrations (2.5 – 20 μM) of MMP inhibitor for 48 hours. Cells were stained and fixed before being analysed using flow cytometry. Two primary antibodies were used to stain Endo180 at different state: (A)A5/158 stains all Endo180 on cell surface, and (B) 39.10 stains Endo180 in single molecular form on cell surface. Relative Endo180 level was calculated by normalising the fluorescent intensity of each treatment group to that of the control cells (Endo180 level set as 100%). Results were represented as mean ± SEM, n = 3 – 6. Nonparametric Kruskal-Wallis test was used to test the significance of differences between treatments. $P \leqslant 0.05$ was considered as significant difference (marked red). Student's t-test was used to compare the significance of the change in a specific treatment group with the control, * $P \leq 0.05$, ** $P \leq 0.01$.

7.3.1.2 The effect of Endo180 expression on MMP-2 / -9 activities

Previous studies showed that MCF-7, which is a less invasive breast cancer cell line, expresses very small amount of MMP-2 and MMP-9 (Bartsch et al., 2003, Kohrmann et al., 2009). Interestingly, the expression level of Endo180 is also lower than detectable amount (Western blot) in MCF-7 cells (Wienke et al., 2003). In this study, we used gelatin zymography to investigate the activities of MMP-2 and MMP-9 in normal MCF-7 cells and Endo180 overexpressing MCF7-E cells. We also investigated whether bisphosphonate treatment could influence MMP-2 or MMP-9 activity in these cells. The results showed that MCF7-E cells had higher MMP-2 and MMP-9 activities than non-transfected MCF-7 cells, especially with MMP-2, which was hardly detectable in non-transfected MCF-7 cells, while bisphosphonates did not show a significant influence in the MMPs activities (Figure 7.7).



Band intensity

MCF-7						
	1	2	3	4	5	6
MMP-9	7981569	5071110	6579489	5234178	5103749	3956177
MMP-2	–	–	–	–	–	–
MCF7-E						
	1	2	3	4	5	6
MMP-9	35520520	22934100	19720260	19316500	22908620	22292340
MMP-2	1471400	1563100	1431920	1314460	1993180	1399440

Figure 7.7 Comparison of MMP-2 / -9 activities between MCF-7 and MCF7-E with/without bisphosphonate treatment

MCF-7 and MCF7-E cells were treated in serum-free conditioned medium that contains 30 – 100 μM of alendronic acid or zoledronic acid. After 48 hours, conditioned medium was collected and analysed using gelatin zymography, to compare activities of MMP-2 / -9 between treatment groups and control groups. This figure shows a representative image of the gelatin zymography in both cell lines, together with the densitometry data. L – Ladder, 1 – no treatment, 2 – alendronic acid 30 μM, 3 – alendronic acid 100 μM, 4 & 5 – zoledronic acid 30 μM, 6 – zoledronic acid 100 μM.

7.3.2 Indirect co-culture of tumour cells and osteoblasts

To investigate whether the contact between osteoblasts and tumour cells could affect the release of Endo180 from both cell types, we designed *in vitro* indirect co-culture experiments using breast / prostate cancer cells and osteoblasts, and measured the level of Endo180 released into the conditioned media, as well as the level of Endo180 on cell surface. In comparison to direct co-culture, indirect co-culture makes it possible for us to distinguish the Endo180 released by each cell type, and to easily measure the level of Endo180 on the surface of different cells. The co-culture was set up in a bi-directional manner, which means that the tumour cells were cultured in the conditioned medium from osteoblasts, while osteoblasts were cultured in the conditioned medium from tumour cells.

Before co-culture, the conditioned medium was collected and analysed using Endo180 ELISA to measure the level of Endo180 (A). After co-culture, used medium was collected and the level of Endo180 (B) was measured again. Control experiment was set up by culturing the same line of cells in fresh media (same type as conditioned media) under the same growth conditions. The level of Endo180 in the medium from control experiment (C) was also measured after the same length of time as in the co-culture experiment. The percentage changes of Endo180 level were calculated as the formula below:

$$\textbf{percentage change} = \frac{(\boldsymbol{B} - \boldsymbol{A}) - \boldsymbol{C}}{\boldsymbol{C}} \times \mathbf{100}\%$$

The percentage change between -100% and 100% represents the level of decrease (-100% – 0%) or increase (0% – 100%) on the release of Endo180 from co-cultured cells in comparison to control cells, while the percentage change lower than -100%

indicates that the co-cultured cells actually absorbed the Endo180 in the conditioned medium, rather than release Endo180 into the medium.

As is shown in Figure 7.8, in the co-culture between breast cancer cell MDA-MB-231 and osteoblasts, the release of Endo180 from tumour cells did not show significant change, and the release of Endo180 from osteoblasts showed a decrease of 10% (P = 0.03, t-test). As to the co-culture of prostate cancer cell PC-3 and osteoblasts, the tumour cells less Endo180 was detected in the conditioned medium after co-culture, which means that there was an uptake of Endo180 by tumour cells, instead of release. Osteoblasts showed an increase of 14% in Endo180 release, but was not statistically significant (P = 0.33, t-test).

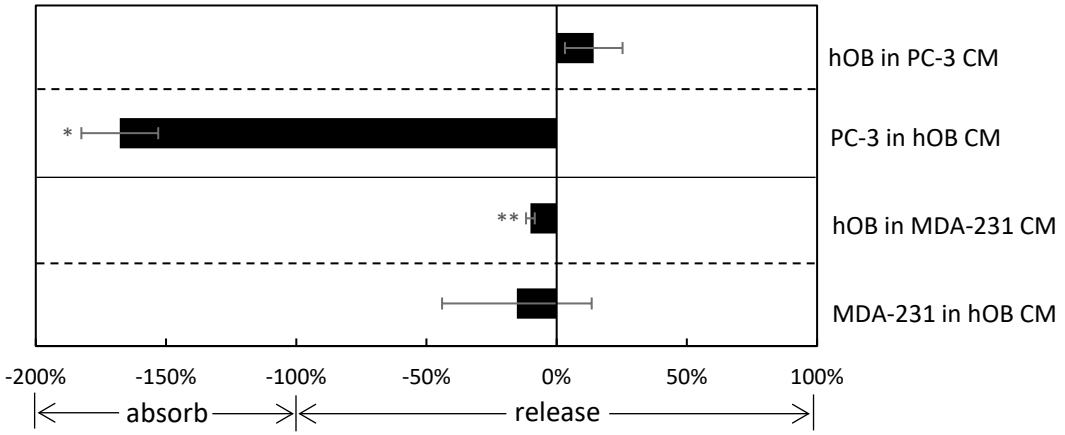


Figure 7.8 The change on the release of Endo180 after indirect co-culture of tumour cells and osteoblasts

Indirect co-culture experiments were carried out between tumour cells and osteoblasts. The level of Endo180 in the conditioned medium were assessed by Endo180 ELISA before and 48 hours after the indirect co-culture. The Endo180 release (or absorb) level was determined by subtracting the level of Endo180 in the conditioned medium before co-culture from the level of Endo180 in the conditioned medium after co-culture. The results were showed as the percentage change of Endo180 release (or absorb) in comparison to the level of Endo180 release in the control group. Results were represented as mean ± SEM, n = 3 – 5. The significance of change was determined by t-test, * $P < 0.05$, ** $P < 0.01$ was considered as significant change. CM: conditioned medium.

We also analysed Endo180 level on the surface of tumour cells and osteoblasts in the indirect co-culture experiments (Figure 7.9). For breast cancer cell – osteoblast co-culture, there was no statistically significant change in Endo180 level on the surface of

MDA-MB-231, while in osteoblasts there was a 38% increase (P = 0.01, t-test), which fits with the changing pattern of Endo180 release measured by ELISA. As to prostate cancer cell – osteoblast co-culture, an increase in Endo180 level was observed on the surface of PC-3 (27%, P = 0.27, t-test), and a similar increase of surface Endo180 was also observed in osteoblasts (27%, P = 0.01, t-test).

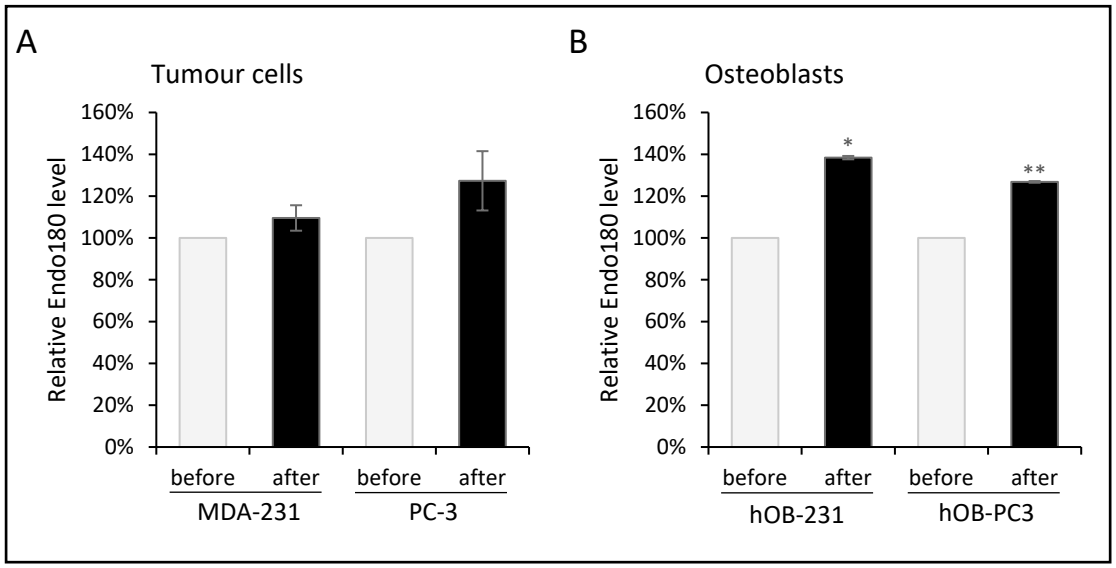


Figure 7.9 Changes of Endo180 on cell surface after indirect co-culture

Endo180 levels on the surface of (A) tumour cells, (B) osteoblasts were assessed following the indirect co-culture. Cells were stained and fixed before being analysed using flow cytometry. Relative Endo180 level was calculated by normalising the fluorescent intensity of each sample to that of the control cells (under the same growth condition and cultured alone). Results were represented as mean ± SEM, n = 2 – 3, * $P < 0.05$, ** $P < 0.01$ by t-test on each treatment group versus non-treatment control group (Endo180 level set at 100%). hOB-231: osteoblasts co-cultured in the conditioned media from MDA-MB-231 cells; hOB-PC3: osteoblasts co-cultured in the conditioned media from PC-3 cells.

We summarised from the co-culture experiments that: 1) indirect contact of breast cancer cells and osteoblasts does not affect the release of Endo180 from tumour cells, but could inhibit the release of Endo180 from osteoblasts, and increase the amount of Endo180 on the surface of osteoblasts; 2) indirect contact of prostate cancer cells and osteoblasts leads to the intake of Endo180 by tumour cells, and also leads to an increase in the level of Endo180 on the surface of osteoblasts.

7.4 Discussion

MMPs are found to be co-functional with Endo180 in collagen internalisation and degradation and are correlated with prostate cancer progression (Kogianni et al., 2009, Ye et al., 2010). Endo180 is also proved to be a novel regulator of the activities of membrane-bound MT1-MMP (Messaritou et al., 2009). In the previous chapters of this study, we found that the release, localisation and expression of Endo180 is regulated by bisphosphonates, and the level of Endo180 in the plasma from breast cancer patients is a potential marker of advanced breast cancer. Moreover, bisphosphonates were shown to be able to inhibit the activity of MMPs in several *in vitro* studies (Teronen et al., 1999, Heikkilä et al., 2002). Based on these findings, we supposed that there might be a relationship between bisphosphonates, Endo180, and MMPs. Our results revealed that the expression of Endo180 in MCF-7 cells lead to an increase of MMP-2 / -9 activity, which was naturally weak in MCF-7 cells (Bartsch et al., 2003, Kohrmann et al., 2009). This finding confirmed our hypothesis on the relationship between Endo180 expression and MMPs activity.

The results of our study did not show a significant effect of bisphosphonates on MMP-2 / -9 activity in MCF7-E cells. This does not indicate that bisphosphonates could not regulate the activity of MMPs in any cell lines, because of the special characteristic MCF7-E. MCF7-E cells are MCF-7 cells that are transfected to overexpress Endo180, so the expression of Endo180 in these cells keeps in a high level. In Chapter 6, we showed that Endo180 expression in MCF7-E cells was not inhibited by bisphosphonates, which was different from un-transfected breast cancer cell line, e.g. MDA-MB-231. Therefore, it will not be surprising that bisphosphonates do not regulate MMP-2 / -9 activity in MCF7-E cells. On the contrary, this ineffectiveness indirectly confirmed that relationship between Endo180 expression and MMP-2 / -9 activity.

We also revealed in the study that MMP inhibitor could inhibit the release of Endo180 from breast cancer cell lines MDA-MB-231 and MCF7-E. Furthermore, the Endo180 localised on the cell surface showed a significant increase in MMP inhibitor treated breast cancer cells. These results, in combination with previous studies, suggest that MMPs could modulate the release of Endo180 from breast cancer cells. We chose MMP-2 and investigated its effect on the release of Endo180, but could not find a correlation between MMP-2 treatment and the change in Endo180 release. As indicated by the results of MMP inhibitor treatment, there should a regulatory relationship between MMPs and Endo180 release. Therefore, we supposed that there might be some other types of MMPs which could regulate the release of Endo180, and different types of MMPs might work together and form a pathway to regulate the release of Endo180 from tumour cells.

Endo180 is known to form a trimolecular complex with urokinase plasminogen activator (uPA) and the urokinase plasminogen activator receptor (uPAR) on the cell surface (Behrendt et al., 2000). Recently, Endo180 is proved to form a complex with CD147, and the Endo180-CD147 complex is found to be indispensable for the stability of the three-dimensional acini formed by prostate epithelial cells, and could also inhibit the EMT of cancer cells, which is essential in cancer progression (Rodriguez-Teja et al., 2015b). The release of Endo180 from the surface of tumour cells could not happen unless the complex formed between Endo180 and uPA/uPAR or CD147 is disrupted. So far, the number studies about the disruption of the complex is still very limited. In our study about the effect of MMP-2 on the release of Endo180 from the surface of breast cancer cells, we coincidentally found that MMP-2 could lead to the disruption of the complex formed by Endo180 and other molecules. The two laboratory-derived monoclonal antibodies (A5/158 and 39.10) that bind to two different epitopes of

Endo180, one of which being the binding domain of Endo180 to form the complex, make it possible to distinguish the Endo180 proteins at single molecular status from those in complex with other molecules. Our results showed that when treated with MMP-2, the level of Endo180 in single molecular form significantly increased, while the total Endo180 level on the surface of tumour cells stayed unchanged. This indicates that MMP-2 could cause the disruption of the complex formed between Endo180 and uPA/uPAR or CD147. In combination with the effect of MMP inhibitor in supressing the release of Endo180, it is supposed that several different types of MMPs might be involved in the regulation of Endo180 release, including the disruption of complex and the shedding from cell surface. Moreover, as bisphosphonates are proven to be able to inhibit bone resorption by abrogating MMP-2 protection induced by plasmin-mediated degradation, it is possible that inhibition of MMPs activities caused by bisphosphonates could stop the disruption of Endo180 complex, so as to promote the stability of epithelial phenotype of tumour cells and inhibit tumour migration (Ichinose et al., 2000). This is also in accordance with down regulation in the level of plasma Endo180 found in bisphosphonates treated breast cancer patients, which indicates that the level of plasma Endo180 could reflect the seriousness or invasiveness of breast cancer.

There are several major drawbacks of the experiments carried out to investigate the effect of MMPs on the release of Endo180 from the surface of breast cancer cells. First, proper control experiments are necessary for the MMP inhibitor treatment experiments. For MMP-2 treatment, the control experiment used in this study to validate the effect of the recombinant MMP-2 protein was gelatin zymography. However, no control experiment was setup for MMP inhibitor treatment. Although the result showed that Endo180 release was modulated by MMP inhibitor treatment, the lack of a control experiment that could validate the effectiveness of MMP inhibitor on the treated cells

makes the results less convincing. Second, control experiments are also lack for the gelatine zymography in this study. There should be a well for negative control (loading buffer) and a well for positive control (lysate of MMP-2/-9 positive cells) to make the experiment complete.

Breast and prostate cancer cells have high chances to migrate to bone at the advanced stages of cancer, which would cause lesions to the bone tissue (Mundy, 2002). Endo180 is crucial for ECM turnover during the metastasis of tumour cells (Curino et al., 2005). In osteoblasts, Endo180 participate in the osteogenic function through the deposition of type I collagen (Caley et al., 2012). Moreover, Endo180 is proved to be a key factor in a novel bone degradation pathway in primary bone cancer (Engelholm et al., 2016). As Endo180 is expressed in tumour cells and osteoblasts, it is possible the change of plasma Endo180 in breast cancer patients is a result of the change in the release of Endo180 from these types of cells, and the contact between tumour cells and osteoblasts might be the reason to the change of release.

The indirect co-culture of tumour cells and osteoblasts showed that: (1) the factors in the conditioned medium from osteoblasts do not influence the release of Endo180 from breast cancer cells, as well as the level of Endo180 on cell surface; (2) osteoblasts showed a decrease in the release of Endo180 when co-cultured with breast cancer cells, and an increase in the level of Endo180 on cell surface; (3) prostate cancer cells cultured co-cultured with osteoblasts caused a decrease of Endo180 level in the conditioned medium, which indicates that the Endo180 protein in the conditioned medium could either bind to the surface or go across the membrane of prostate cells. Accordingly, the level of Endo180 on the surface of prostate cells showed an increase in co-culture experiments; 3) when co-cultured with prostate cells, osteoblasts showed an increase in

the level of Endo180 on cell surface, and the level of End180 released into the conditioned showed a non-significant increase.

Our results showed different regulatory patterns in breast cancer cell – osteoblast co-culture and prostate cancer cell – osteoblast co-culture, which is in accordance with the different patterns of bone effects caused by metastasis breast cancer and prostate cancer. It is known that the end result of metastasis by breast and prostate cancer is usually different, as metastatic breast cancer always leads to osteolytic lesion, and metastatic prostate cancer often cause osteoblastic lesion (Mundy, 2002). In this study, an upregulation of the release and surface level of Endo180 were observed in osteoblasts with the co-culture of both breast tumour cells and prostate tumour cells. Although increase in the release and cell surface level of Endo180 could neither indicate a certain upregulation of Endo180 expression in osteoblasts nor prove the enhancement of the osteoblastic function, this is still a potential reflection of the increased collagen deposition of osteoblasts. However, the actual type of the lesion to bone caused by the tumour cells is a result of the complex functional interfere between the invaded tumour cells, osteoblasts, osteoclasts, and the local environment, the over-release of Endo180 and other factors from osteoblast might work as a trigger to the function of osteoclasts, which could lead to an osteolytic lesion instead (Mundy, 2002). The increase in Endo180 on the surface of osteoblasts might also be an indicator of the responsive local bone-formation in the breast cancer cell invaded niches (Stewart et al., 1982). On the other hand, our results showed that the indirect contact with osteoblasts led to different regulation patterns to breast and prostate tumour cells on the release of Endo180 from cell surface, this also indicates the different interactions between the two types of tumour cells and osteoblasts.

8 Final Discussion

Breast cancer is a heterogeneous disease characterized by changes in the levels and activities of important cellular proteins (Cole et al., 2013). These changes also provide a viable way for the detection of tumour occurrence by measuring the marked changes in the level of an altered proteins. This is the reason for measuring Endo180 levels in the plasma of breast cancer patients as a biomarker for metastatic disease. Endo180 plays important roles in collagen turnover, as well as in the migration and metastasis of tumour cells, and is shown to be closely correlated with EMT in tumour progression (East et al., 2003, Sturge et al., 2003, Wienke et al., 2003, Kjøller et al., 2004, Sturge et al., 2006, Wienke et al., 2007, Caley et al., 2012, Rodriguez-Teja et al., 2015b). Furthermore, it has been demonstrated that: 1) the ectodomain of Endo180 was released from breast cancer cells in a soluble form, 2) the level of plasma Endo180 was markedly higher in patients with recurrent / metastatic breast cancer than early breast cancer patients, and 3) breast cancer patients with bone metastatic disease who had received bisphosphonate treatment showed lower plasma Endo180 levels in comparison to the treatment naive patients (Palmieri et al., 2013a).

Therefore, this project was designed to further explore Endo180 as a biomarker for advanced breast cancer, and to explore its correlation with bisphosphonate treatment, as well as to investigate the mechanisms underlying its release. The study consists of four sections: 1) the development of an immunoassay for Endo180 measurement; 2) the investigation of Endo180 as a biomarker of advanced breast cancer; 3) the study of the regulation by bisphosphonates on Endo180 release and expression; 4) the study of the mechanisms under the release of Endo180.

8.1 Endo180 as a potential biomarker for advanced breast cancer

In order to further explore the feasibility of using Endo180 as a biomarker for advanced breast cancer and its potentiality in clinical application, plasma samples were collected from breast cancer patients at early and advanced stage in collaboration with Professor Carlo Palmieri at the University of Liverpool. Endo180 levels in the plasma samples from two cohorts of early and advanced breast cancer patients were analysed, in which the correlation of the Endo180 level with the stage of breast cancer was investigated.

It was observed in this study that the level of Endo180 in advanced breast cancer patients is significantly higher than early breast cancer patients (Figure 5.2). Endo180 was also demonstrated to have high sensitivity and specificity in the receiver operating characteristics analysis to test its capability in distinguishing advanced breast cancer from early breast cancer (Figure 5.5). Within the sample groups used in this study, Endo180 did not show correlation with the pathological characteristics (e.g. age, hormone receptor status) of the patients (Table 5.11, Table 5.12). These results indicate that the level of plasma Endo180 in breast cancer patients is correlated with the advanced stage of the disease, and has the potential to be used as a biomarker for advanced breast cancer.

However, due to the limited number of samples provided for this study, a further and more detailed analysis is necessary to verify the correlation of plasma Endo180 levels with the survival outcome of breast cancer patients, as well as the correlation with therapy response.

We also compared the performance of Endo180 with three conventional tumour markers that were used in breast cancer diagnosis or treatment, CA 15-3, CTX and BAL. The level of Endo180 showed a linear correlation with breast cancer biomarker CA 15-3 and bone resorption marker CTX, which could be explained using the mechanisms

underlying the regulation of these biomarkers that were reported previously: 1) MUC-1 protein, of which CA 15-3 is an epitope, is found to be overexpressed and aberrantly glycosylated in breast cancer cells, and is released into serum (Burchell et al., 2001, Hattrup and Gendler, 2008, Kufe, 2009). The cleavage of CA 15-3 is mediated by MT1-MMP (Thathiah and Carson, 2004). The co-functional relationship of Endo180 and MT1-MMP suggests a possible correlation between the serum level of the two markers (Kogianni et al., 2009, Ye et al., 2010, Lund et al., 2012); 2) As enzymatic hydrolysis products of collagen I, the level of CTX is correlated with the level of collagen degradation, which makes serum CTX levels an indicator of the bone resorption level (Christgau et al., 2000). Given the ability of Endo180 in binding to the C-terminal region of collagen I and its important role in ECM turnover in cancer, it is postulated that the level of Endo180 level could be correlated with the level of CTX in ECM (Curino et al., 2005, Thomas et al., 2005) (Figure 8.1).

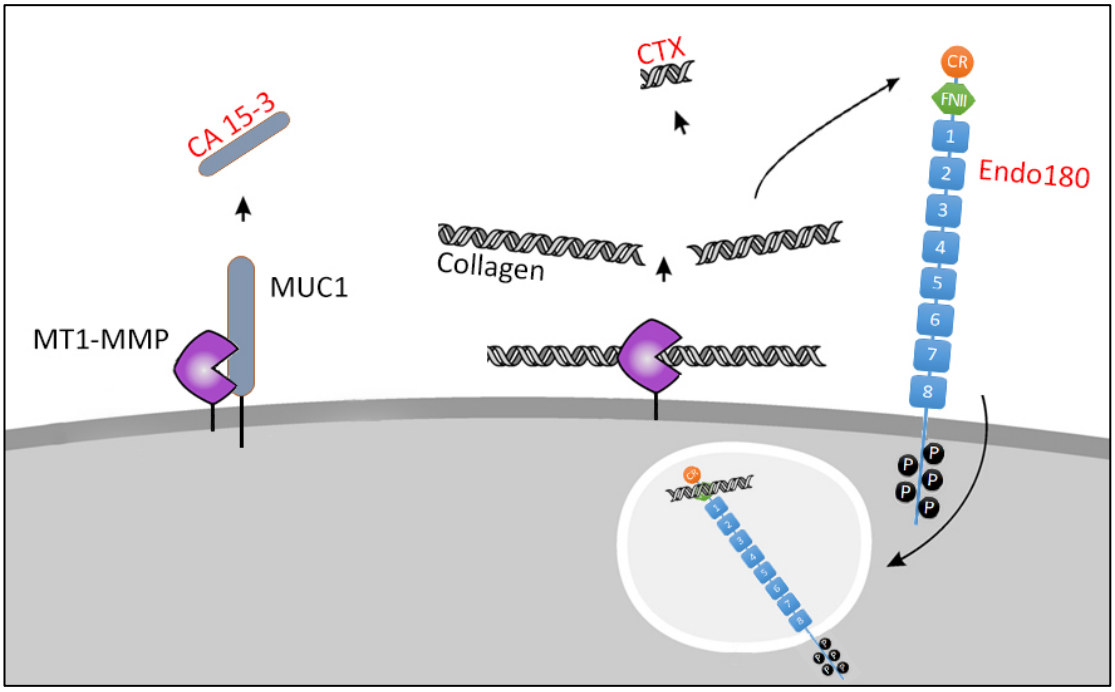


Figure 8.1 The potential correlation between Endo180, CTX and CA 15-3

As a prerequisite for tissue remodelling, ECM degradation takes place during cancer invasion. MT1-MMP and Endo180 co-functions in this procedure: MT1-MMP could cleave collagen in ECM (which could lead to the release of CTX), and the collagen fragments could then be internalised by Endo180. MT1-MMP could also mediate the cleavage of MUC1 from cell surface, the serum level of which could be measured as biomarker CA 15-3. Adapted from Lund et al. (2012) under CC-BY licence.

Initially, we planned to investigate Endo180 as a dynamic indicator that responds to bisphosphonate treatment in advanced breast cancer patients with bone metastasis. However, the serial collection of plasma sample from eligible breast cancer patients was not implemented by our collaborator, as a result, the Endo180 measurement and subsequent analysis could not be carried out. Another drawback of this study is that there was no follow-up information update of patients' status, regarding disease progression and the recurrence of cancer. By comparing the original plasma Endo180 level and the patient's current status, we could further confirm the capability of Endo180 to predict the occurrence of metastasis in early breast cancer patients. Further investigation could focus on the dynamic response of Endo180 to bisphosphonate treatment, and a proper follow-up study into the capability of Endo180 in predicting the occurrence of metastasis in early breast cancer patients.

It was concluded from this study that Endo180 is a potential biomarker for advanced breast cancer. Our preliminary data also suggested a correlation between the increased plasma Endo180 level with the level of related biomarkers such as CA 15-3 and CTX in advanced breast cancer patients. However, the mechanism and regulatory factors of Endo180 release had yet to be discovered, which led to the implement of the subsequent studies in Chapter 6 and 7, for a further understanding of the regulation of Endo180 release / expression, and its correlation with tumour metastasis.

8.2 Bisphosphonates modulate the expression and release of Endo180

A number of clinical studies have shown that using bisphosphonates as an adjuvant treatment could not only relieve the bone pain and skeletal complication of bone metastases but also reduce the risk of skeletal-related events, prevent or delay the occurrence of bone metastases, and improve the patients' survival rate (Brown and Coleman, 2002, Gnant et al., 2011, Palmieri et al., 2013b, Early Breast Cancer Trialists'

Collaborative Group, 2015). The antiresorptive effect of bisphosphonates on osteoclasts has been well described, while other targets of bisphosphonates such as osteoblasts, angiogenesis and the immune system are attracting more and more attention (Bukowski et al., 2005). Besides the antiresorptive effect, more studies are focusing on the direct anti-tumour effect of bisphosphonates on tumours cells, and it has been found that *in vitro* treatment of breast cancer cells with bisphosphonates could inhibit cell growth, cell invasion and their adhesion to bone (Senaratne and Colston, 2002, Verdijk et al., 2007). Moreover, advanced breast cancer patients who had been administered bisphosphonates showed lower plasma Endo180 levels in comparison to bisphosphonates naive patients (Palmieri et al., 2013a). This suggests a correlation between Endo180 and the anti-tumour effect of bisphosphonates. In this study, *in vitro* bisphosphonate treatments with tumour cells and osteoblasts were set up to investigate its effect on Endo180 expression and release.

As was revealed by the treatment, Endo180 expression in breast cancer cells (except Endo180 overexpressing MCF7-E cells) was inhibited by bisphosphonates. Correspondingly, the level of Endo180 released into cell culture medium, as well as the level of Endo180 on the cell surface, both decreased under bisphosphonate treatment (Figure 6.3, Figure 6.10). The result indicated that the decrease of Endo180 release level, as well as cell surface level, are resulted from the inhibition of Endo180 expression by bisphosphonates, which might be an essential part of their anti-tumour effects. However, further investigations are still in need to validate the effect of bisphosphonates on Endo180 expression in tumour cells, as well as the correlation between this effect and the anti-tumour effect of bisphosphonates. For example, the dose range of bisphosphonates used in the drug treatment was relatively small (3 – 100 μM). The half-time of bisphosphonates in the circulation is short, and the distribution

of the drug throughout the body is uneven (Lin, 1996, Cremers and Papapoulos, 2011). Therefore, wider drug concentration and treatment duration ranges are necessary to accurately reflect the treatment that cells receive *in vivo*.

Besides the antiresorptive effect on osteoclasts, bisphosphonates were also shown to have effects on osteoblastic cells, such as enhancing mineralisation and alkaline phosphatase production, inhibiting osteoblast apoptosis, promoting osteoblast differentiation, and causing the cleavage of transmembrane RANKL (Igarashi et al., 1997, Plotkin et al., 1999, Abe et al., 2000, D'Aoust et al., 2000, Pan et al., 2004). Is has also been reported that zoledronic acid caused a significant reduction in the number of osteoclasts and osteoblasts in trabecular bone *in vivo*, and breast cancer cells arriving in this modified bone microenvironment appeared to preferentially locate to osteoblast-rich area (Haider et al., 2014). This suggests that osteoblasts may be key components of bone metastasis in breast cancer, and is a potential therapeutic target of bisphosphonates. Here in this study, we also investigated if bisphosphonates influence the release and expression of Endo180 in osteoblast and osteoblast-like cells. The results showed that bisphosphonate treatment could cause an increase on Endo180 expression and release in osteoblasts (Figure 6.4, Figure 6.7), while the level of Endo180 on cell surface was not significantly changed (Figure 6.11). As to osteoblast-like U-2 OS cells, the changes were similar to osteoblast under low concentrations of bisphosphonates (30 μM), while at high concentration (100 μM), there seemed to be a significant reduction in cell number, which was confirmed not to be apoptosis in TUNEL assay. We supposed that as an osteosarcoma cell line, U-2 OS might have different response to bisphosphonates at high/low concentrations: at low concentrations, the effect of bisphosphonates on U-2 OS is more like the effect on osteoblasts; at high concentrations, the effect is more like on tumour cells. These results suggest that there

is no significant inhibition of Endo180 expression / release by bisphosphonates in osteoblasts.

In combination with the previous revealed anti-tumour effect of bisphosphonates, we postulated a comprehensive anti-tumour effect of bisphosphonates on the tumour invaded bone microenvironment. The effect of bisphosphonates on the local niche, where breast cancer cells have invaded and broke the original balance in bone resorption and production, is a multi-pronged approach to the restoration of the initial balance (Figure 8.2). The first key effect is the antiresorptive effect on osteoclasts, which leads to the inhibition of bone degradation. This also reduces the expression of growth factors that could assist the localisation of tumour cells to the bone as an important part of the "vicious circle", so as to supress the progression of bone metastasis (Roodman 2004, Ottewell et al., 2015). The second effect is the direct anti-tumour effect of bisphosphonates on tumour cells, which include: 1) the inhibition of tumour growth as a result of the induction of apoptosis to tumour cells (Tassone et al., 2003, Verdijk et al., 2007); 2) the inhibition of Endo180 expression, which leads to the inhibition of collagen degradation by tumour cells and the suppressed adhesion of tumour cells to ECM (Senaratne et al., 2002, Pickering et al., 2003); 3) other inhibitive effects on key proteins such as inhibiting the activities of MMPs (Boissier et al., 2000, Teronen et al., 2000). The third effect is the protective effect on osteoblasts as described before. All these effects might work simultaneously and collaboratively, which as a result break the "vicious circle" formed between tumour cells and the bone cells, and restores the normal balance between bone resorption and formation.

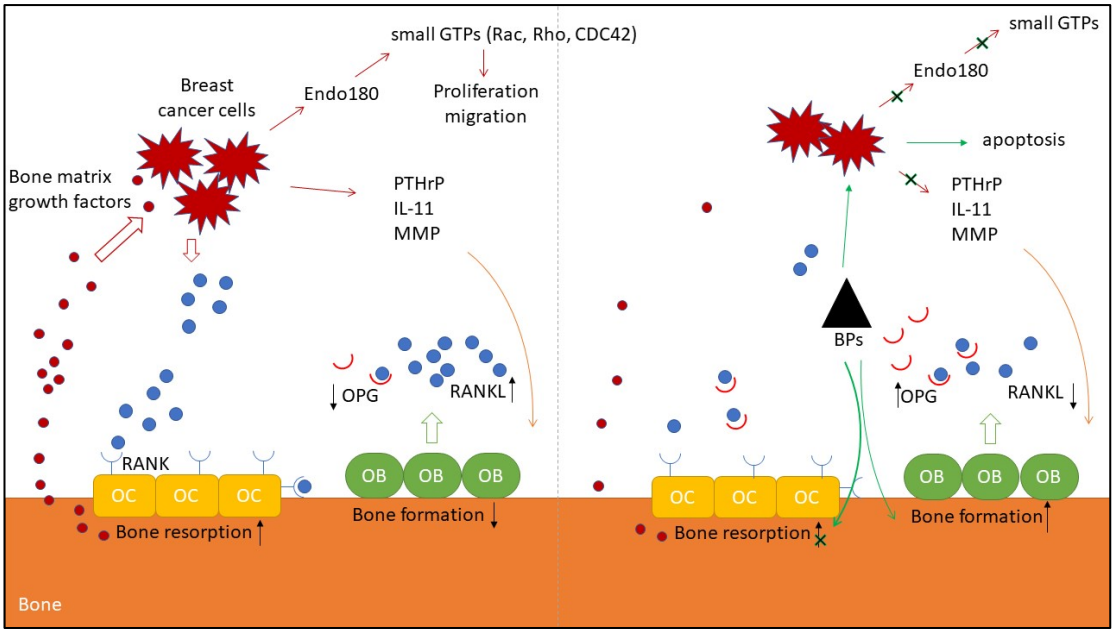


Figure 8.2 The changes caused by invasive breast cancer cells in bone and the potential anti-tumour effects of bisphosphonates

Breast cancer cells in the bone microenvironment express parathyroid hormone-related protein (PTHrP), cytokines and growth factors that negatively impact osteoblast function. They also express Endo180 and MMPs to facilitate the migration and proliferation. OPG expression by osteoblasts are inhibited, which leads to more RANKL are able to bind to RANK so as to promote the formation and function of osteoclast. Bone resorption releases growth factors from the bone matrix that promotes tumour growth, which in turn promotes osteoclast activity and bone resorption, thus form a "vicious circle". Bisphosphonates could break the "vicious circle" by supressing the activities of osteoclasts, inhibit tumour growth, inhibit the expression of Endo180 and other growth factors by tumour cells, and restore the function of osteoblasts.

8.3 The mechanisms under Endo180 release and its changes in breast cancer

Previous study revealed that Endo180 is released by breast cancer cells MDA-MB-231 and MCF-7 (Palmieri et al., 2013). However, little is known about the mechanism underlying the release of Endo180, the proteins participating in this procedure, and the factors that cause the changes in plasma Endo180 levels in breast cancer patients. Here in this study, we investigated two factors that we hypothesised to mediate the cleavage of Endo180 from the cell surface and lead to the change in Endo180 release: 1) indirect contact between tumour cells and osteoblasts, and 2) MMPs.

In bone metastasis, the interaction between tumour cells and local bone cells is a key influencing factor on the invasion of tumour cells. As is mentioned in Section 8.2, the

interplay between breast cancer cells and osteoclasts could form a “vicious circle”, in which the growth factors released by tumour cell would promote the formation and function of osteoclasts, while the enhanced osteoclast formation cause more growth factors being released which in turn promote the growth of breast cancer cells (Roodman 2004). It is also proved that the promotion in osteoclast formation is a result of the interaction between breast cancer cells and osteoblasts by the regulation of RANKL expression in osteoblasts (Thomas et al., 1999). Based on the interactions between tumour cells and osteoblasts / osteoclasts, we supposed that the interaction between invasive tumour cells and osteoblasts might also influence the release of Endo180 on both cell types. Therefore, indirect co-culture experiments were set up in this study to the influence on Endo180 release. Our results showed that the indirect contact between breast cancer cells and osteoblasts caused an increase in the release of Endo180 from osteoblasts, while breast cancer cells were not significantly influenced. These results indicate that the invasion of breast cancer cells to bone or other metastasis sites does not lead to the increase in Endo180 release from the invasive breast cancer cell. The increase of plasma Endo180 could be resulted from the increase on Endo180 release from tumour cells during the EMT of tumour cells at the beginning of metastasis. In that case, the plasms level of Endo180 of breast cancer patient would be a sensitive early indicator of the occurrence of invasive cancer cells.

Another factor that we hypothesised to play a role in changing plasma Endo180 levels is the cleavage of Endo180 by MMPs, based on the correlation between Endo180 and MMPs reported previously, especially MT1-MMP, one of the membrane-bound MMPs that mediate the activation of MMP-2 (Madsen et al., 2007, Wagenaar-Miller et al., 2007, Kogianni et al., 2009). Bioinformatic modelling has predicted an MMP-2 cleavage site on the transmembrane domain of Endo180. Therefore, it is suggested that

MMPs are functional on the cleavage of Endo180 from cell surface. In this study, we used MMP-2 and a broad-spectrum MMP inhibitor, Marimastat, to investigate if MMPs could affect the release of Endo180 in breast cancer cells. Our results revealed no significant change on the release of Endo180 in breast cancer cells treated with MMP-2, which suggests that MMP-2 alone could not cleave Endo180 from the cell surface. However, flow cytometry on breast cancer cells stained with two different anti-Endo180 monoclonal antibodies revealed that, when treated with MMP-2, there was an increase on the level of non-complexed (single molecular) Endo180 on the cell surface, while the total level of Endo180 remained the same. This indicates that MMP-2 could lead to the disintegration of the complex formed between Endo180 and other molecules, such as CD147 and uPA/uPAR. It was also found that when treated with MMP inhibitor, the level of Endo180 released into the conditioned medium showed a significant decrease, and correspondingly, the level of Endo180 on the cell surface was increased under MMP inhibitor treatment. These results suggest that the inhibition of MMPs could supress the release of Endo180 in breast cancer cells. Therefore, it is possible that one or more types of MMPs are functional in mediating the cleavage of Endo180.

Bisphosphonates were found in previous studies to be able to inhibit MMPs so as to supress the migration of tumour cells, and could regulate the release of MMP-2 from human osteoblasts (Teronen et al., 1999, Ichinose et al., 2000, Heikkilä et al., 2002). Moreover, Endo180-CD147 complex is proved to be essential in maintaining the epithelial phenotype of prostate epithelial cells (Rodriguez-Teja et al., 2015b). It was recently revealed that matrix stiffness up-regulates the activities of MMPs, and the inhibition of MMPs significantly reduces angiogenic outgrowth of endothelial cells in stiffer crosslinked gels (Bordeleau et al., 2017). Taken together, it is supposed that: 1) the inhibition of MMPs by bisphosphonates could be correlated with the inhibition of

Endo180 release by bisphosphonates in tumour cells; 2) the released MMP-2 from tumour cells could disrupt the Endo180-CD147 complex on cell surface, and this could be supressed by bisphosphonates as one of their anti-tumour effects; 3) the inhibition of MMPs by bisphosphonates and the subsequence suppression on tumour migration might be an important anti-tumour effect of bisphosphonates to prevent the invasion of tumour cells to metastatic sites.

In combination with the findings in the co-culture experiments and previous studies, we concluded that the release of Endo180 could be a result of tumour progression and an indicator of the EMT of tumour cells (Figure 8.3): The formation and expand of tumour cells at the primary site could lead to physiological changes of the local environment, e.g. hypoxia. Hypoxic condition would trigger the expression of lysyl oxidase (LOX) by tumour cells (Cox et al., 2015). LOX-mediated collagen crosslinking increases insoluble matrix deposition, tissue tensile strength and matrix stiffness (Payne et al., 2007). The increased stiffness could in turn intensify the hypoxia condition and stimulate the secretion of LOX by tumour cells, hence forms a vicious circle. On the other hand, increased matrix stiffness could trigger the expression of essential matrix turnover proteins by tumour cells, such as MMPs, integrin and Endo180, which would trigger EMT and promote the migration and invasion of cancer cells (Billottet et al., 2008, Levental et al., 2009, Kessenbrock et al., 2010, Canesin et al., 2015, Rodriguez-Teja et al., 2015a, Caley et al., 2016, Bordeleau et al., 2017). In turn, EMT could stimulate the expression of Endo180, which could be the direct cause of the increased plasma Endo180 level in breast cancer patients (Ikenaga et al., 2012).

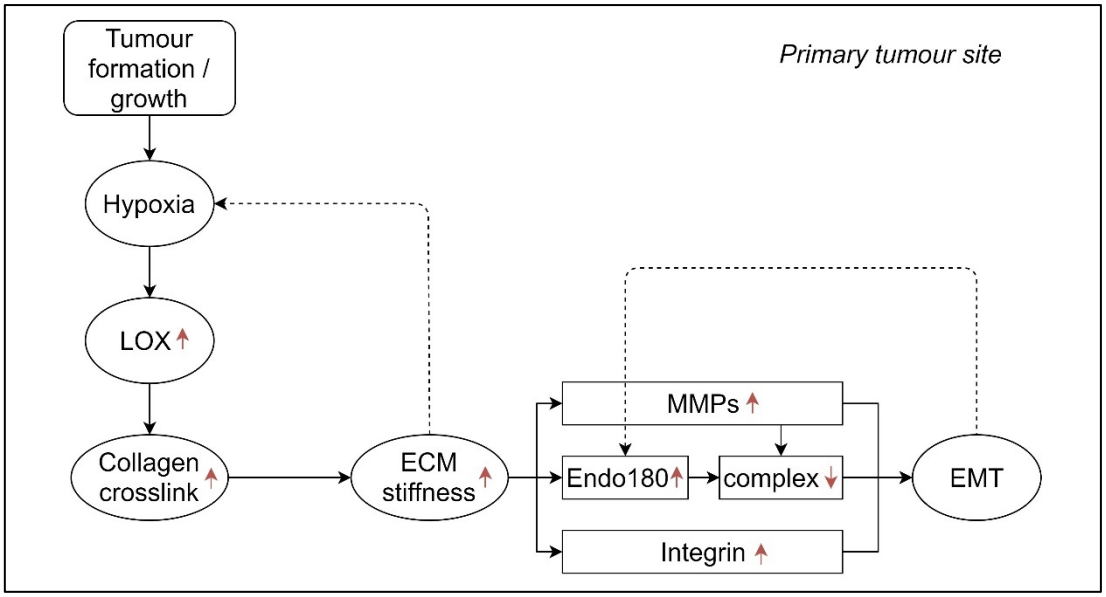


Figure 8.3 Potential mechanisms under the release of Endo180 during tumour progression
Tumour growth causes hypoxia, which triggers LOX expression and LOX-mediated collagen crosslinking, hence increases matrix stiffness. The increased stiffness could in turn intensify the hypoxia condition. Increased matrix stiffness could trigger the expression and functions of MMPs, integrin and Endo180 in tumour cells, which would trigger EMT of cancer cells. MMPs could also break the Endo180-CD147 complex (EMT suppressor), and mediate the release of Endo180 from tumour cells. The mesenchymal phenotype of cancer cells could in turn stimulate the expression of Endo180.

8.4 Establishment of ELISA for Endo180 quantification

At the beginning of this project, an ELISA for Endo180 quantification was established in order to facilitate the implement of Endo180 measurement in the subsequent clinical study and mechanism research.

It was necessary to develop a highly sensitive, accurate and efficient assay for Endo180 measurement to replace the previously used protein immunoblot. In comparison to protein immunoblot, the in-house ELISA has the following advantages: 1) Accurate. ELISA is a quantitative assay, in which the absolute concentration of the target protein could be determined by comparing the plotting the OD readings of the unknown samples onto the dose response curve of standard protein. In contrast, protein immunoblot is a semi-quantitative assay that could be only used to compare the differences between samples. 2) High-throughput. In a standard ELISA, up to 40 samples could be loaded in duplicate onto one microplate. In comparison, protein

immunoblot assay could only process 9 – 14 samples in a standard polyacrylamide gel with no duplicate. 3) Efficient. A standard sandwich-type ELISA usually takes about five hours to finish, which can be done within one day, while a standard protein immunoblot with four gels in one tank usually takes more than eight hours, plus overnight incubation (Table 8.1).

Table 8.1 Comparison between in-house ELISA, protein immunoblot, and commercial ELISA

	In-house ELISA	*Protein immunoblot*	*Commercial ELISA*
Advantages	1. sensitive 2. quantitative 3. high-throughput 4. efficient 5. cost-effective	1. sensitive 2. mature technique	1. same advantages as in-house ELISA 2. ready to use
Disadvantages	1. have to spend time in development and validation 2. need experienced operators	1. semi-quantitative 2. low-throughput 3. time-consuming	1. high cost 2. performance not stable between batches 3. risk of getting unreliable product

Before deciding to developing the ELISA for Endo180, we have taken a survey about the Endo180 ELISA kits that were commercially available. We found some on-line suppliers who claimed to produce ELISA kits for Endo180/MRC2, but none of them were major suppliers, and the detection/reactivity ranges of the kit varied between suppliers. This caused our suspicion on the authenticity and reliability of these products. Moreover, the listed prices of the commercial kits were very high. Taking into account the above concerns, plus the fact that in this project there would be a large number of samples to be measured (without considering the potential repetitions), we decided that it was necessary to establish our own ELISA for Endo180 quantification. With the availability of two strains of mouse hybridomas that could produce the two anti-Endo180 monoclonal antibodies, A5/158 and 39.10, and the transfected breast cancer cell line MCF7-E to express Endo180 protein, which could be used as standard protein,

together with the mature techniques in protein isolation and purification, it was possible to establish a reliable ELISA for Endo180 quantification.

Despite the advantages in many aspects such as effectiveness, sensitivity and cost, the in-house Endo180 ELISA have some disadvantages as well. First, there was no ready to use antibody pair and standard protein for Endo180 ELISA, the preparation of which made the whole development procedure more complicated. However, once produced, the amount of antibodies and standard protein would be sufficient for a number of trials ($\geq$ 50 plates). Second, the operator of the assay needs to be well trained and experienced to carry out the procedure properly, in other words, the performance of the assay is highly dependent on the experience and skills of the operator. This problem could be overcome by making an operation manual which contains the operating procedures and tips, but the beginners might still need practices before mastering all the procedures. Third, if the assay is to be commercialised, the two antibodies and standard protein will need to be mass produced, in order to further reduce the complexity and cost. A better way to produce and quantify the standard protein will be necessary to achieve this goal.

As can be seen in Chapter 4 and the practical applications showed in other chapters, the in-house Endo180 ELISA could accurately measure and compare the levels of Endo180 in different types of samples, including patient plasma and cell culture media. The Endo180 protein collected and purified from MCF7-E cells also performed well, and a linear dose response curve was generated from serial dilutions of the standard protein (Figure 4.7). In conclusion, as the first step of this project, a highly accurate and sensitive ELISA was established for the measurement of Endo180 in different research and clinical samples, which lay the foundation for the rest studies of the project.

8.5 Recommendations for future study

Further investigation into the clinical implement of Endo180 as a biomarker for advanced breast cancer is necessary. In this study, we have revealed that plasma Endl180 level in breast cancer patients is correlated with disease stages, and the high plasma Endo180 level indicates poor prognosis of patients. However, due to the limitation of sample size and type of patients available, we were not able to carry out the follow-up analysis of the changes in plasma Endo180 levels during the progression of breast cancer, as well as the dynamic response of plasma Endo180 levels to bone therapy. A further validation of Endo180 in larger size of patients with known outcome and metastatic status is in need. These could be explored in future studies where completed serial collections of breast cancer patient samples are available. Moreover, it is also necessary to investigate the implement of Endo180 as a biomarker for other tumour types.

As to the mechanism investigation of Endo180 release, *in vitro* experiments were carried out in this study, and the results have revealed the effect of bisphosphonates on the expression and release of Endo180 in tumour cells and osteoblasts. As the migration of tumour cells to the bone tissue is a comprehensive procedure which involves the interactions between different cell types and the local environment, it is necessary to investigate the mechanism *in vivo*, which could reflect the actual procedures that are happening in the bone niche. Although MMP-2 are shown not to cleave Endo180 from tumour cells directly, MMP inhibitor experiments demonstrated that other MMPs could play potential roles in Endo180 cleavage. It would be interesting to find out which MMP(s) could mediate the cleavage of Endo180 from cell surface, and the regulation of the process.

8.6 Final remarks

In conclusion, an in-house ELISA was successfully established in this study, and was shown to be accurate, efficient, and cost-effective. Using this method, we measured the plasma Endo180 levels in early breast cancer patients and advanced breast cancer patients, and revealed the correlation between plasma Endo180 level and the stage of breast cancer. Due to the limited number and types of patients, we were not able to analyse the dynamic change of plasma Endo180 level in tumour progression and throughout bone therapy, which could be a potential interest for future studies.

For the first time, the mechanistic study was carried out to investigate the release of Endo180, the factors that mediate Endo180 release and the cause of the change of Endo180 release level during tumour progression. We also investigated the effect of bisphosphonates on the release / expression of Endo180 as a novel anti-tumour effect. The combination of the data collected in this study and the finding in previous research revealed a hypothesised pathway of how the level of Endo180 is changed during tumour progression: 1) The formation and expansion of the primary tumour lead to tissue stiffness, which upregulates the activities of essential matrix turnover proteins (MMPs, integrin, Endo180) in the local niche (Levental et al., 2009, Kessenbrock et al., 2010, Caley et al., 2016, Bordeleau et al., 2017). The changes in MMPs activity and could cause the breakage of Endo180-CD147 complex and the release of Endo180, which could trigger the EMT of tumour cells together with other aberrant expressed ECM factors (Galliher and Schiemann, 2006, Billottet et al., 2008, Canesin et al., 2015). The mesenchymal phenotype of tumour cells could further enhance the upregulation of Endo180 expression, which would lead to the increase of Endo180 release (Ikenaga et al., 2012). At this stage, the measurement of plasma Endo180 level in patient could reveal the occurrence of invasive cancer cells and predict tumour migration. 2) When

breast cancer cells migrate to bone, a metastatic micro-niche was formed between the invaded tumour cells and bone cells, where a “vicious circle” would establish and cause a continuous bone lesion. Bisphosphonates could break the “vicious circle” by multi-pronged suppression of tumour cells and osteoclasts, including the inhibition of End180 expression and other growth factors, such as MMPs, in tumour cells, which would inhibit tumour cell proliferation and migration.

Lastly, the measurement of plasma Endo180 and the immunoassay developed in this study have the potential to be used as a screening tool in breast cancer or other cancer types. This potential must be further validated in future clinical trials and mechanism studies. Endo180, which is expressed by tumour cells and plays a key role in cell migration and bone turnover, could also be a potential target in tumour therapy.

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