Aus der Klinik für Rheumatologie

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

The Role of Interleukin-6 in Breast Cancer Cell Growth in Bone

zur Erlangung des akademischen Grades

Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät

Charité – Universitätsmedizin Berlin

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Datum der Promotion: 03.06.2012

Acknowledgements

For helping me directly in my attempts to complete my study I wish to express my deepest gratefulness to the following people:

- I am dearly indebted to my supervisor Dr Yu Zheng for his excellent guidance, encouragement, timeless effort and infinite patience throughout my research. I am very appreciative of him sharing his wisdom and great academic knowledge, providing steady support, understanding, and investing of his valuable time. I highly value Dr Zhengs technical help, care and support without which this work would not have been possible.
- My most profound thanks to Dr Hong Zhou who has guided me throughout my work and given new incentives, encouragement and provided technical help.
- I wish to deeply thank Prof. Markus J. Seibel and Prof. Frank Buttgereit for organizing the exchange program that made it possible for me to pursue my study at the ANZAC research institute. Moreover, their help and critical comments and suggestions for my project have been very helpful in finishing this work.
- Great thanks to Dr Colin Dunstan for his comments, advice, suggestions and help in finishing this thesis.
- Many thanks go to the laboratory manager, Mr James Modzelewski, for his time and support, especially in regard to technical assistance.
- Many thanks to all of members of the Bone Research Program. Particularly, Dr Robert Kalak for his contribution and help with microCT scanning and imaging. Ms Colette Fong-Yee, Ms Li Laine Ooi, Ms Janine Street, Dr Markus Herrmann, Mr Holger Henneicke for their generosity, friendship and of course help in any questions that arose during the course of my study.
- I wish to dearly thank Mr Mamdouh Khalil and his excellent animal house staff, who looked after my mice and made all experiments possible.
- Many thanks to Dr Timo Gaber for his assistance in statistics without which analysing the results would not have been possible.
- Greatest thanks to my friends Christopher Rudolf for his patient help and support in formatting and Natalie Farrawell and Michael Rose for her linguistic and editorial assistance.

- I also wish to thank the Bone Research Program at ANZAC Research Institute, the Dept of Endocrinology at Concord Hospital, and my own University, the Charité Berlin, for providing financial support through a scholarship. Without these, the course of my work would have been impossible.

Abstract

Breast cancer metastasizes preferentially to bone where it causes mostly osteolytic lesions. Interleukin-6 (IL-6) plays a major role in the response to injury or infection and is involved in the immune response, inflammation and haematopoiesis. Its deregulation impacts numerous disease states, including breast cancer. There is good clinical evidence that high serum IL-6 levels in breast cancer patients correlate with poor prognosis and rapid disease progression. Conversely, several *in vitro* and *in vivo* studies have demonstrated that lower IL-6 levels are associated with reduced cancer cell growth. Therefore, IL-6 seems to be an important player in the development of breast cancer metastasis in bone.

The overarching aim of my research was to better understand the role of IL-6 in breast cancer bone metastasis and breast cancer cell growth. To this end, I used a xenograft model of osteolytic breast cancer metastasis in which IL-6 production by the cancer cells had been reduced through genetic manipulation, or IL-6 receptor signalling had been blocked through administration of a humanized anti-IL-6 receptor (IL-6R) antibody. The following specific aims were defined:

1) To assess the effect of IL-6 knock down in human MDA-MB-231 breast cancer cells on cancer cell growth *in vitro,* and *in vivo* within the bone microenvironment.

2) To investigate the effect of IL-6 knockdown on breast cancer cell growth in a non-skeletal environment (mammary fat pad).

3) To investigate the autocrine effects of IL-6 signalling on MDA-MB-231 breast cancer cell growth *in vitro* and *in vivo*, using the anti-IL-6R antibody, Tocilizumab.

Overall, results were as follows:

1) Interleukin-6 (IL-6) knock down in MDA-MB-231 breast cancer cells through lentiviral transduction lead to a reduction of IL-6 mRNA and protein production by more than 50%. This did not have an effect on MDA-MB-231 cell growth but reduced invasive properties of the cells *in vitro*.

2) Compared to non-target controls, knockdown of endogenous IL-6 production in breast cancer cells by stable shRNA expression led to a significant reduction of cell growth within the bone microenvironment.

3) IL-6 knockdown cells injected into the mammary fat pad of nude mice grew similarly to their non-target counterparts, indicating that the effect of IL-6 on cancer cell growth depends on, and is mediated by, the bone microenvironment.

4) Partial inhibition of autocrine IL-6 signalling in breast cancer cells through treatment with the anti-human IL-6R antibody, Tocilizumab, at low dose resulted in clear, albeit statistically not significant reduction of breast cancer cell growth within the bone microenvironment.

When metastatic breast cancer cells invade the bone microenvironment, the balance of slow and continuous bone turnover is usually disturbed in favour of net bone loss. The vicious cycle is a model of breast cancer metastasis to bone that explains how tumour cells induce matrix degradation through osteoblast and osteoclast activation, which in turn leads to the release of growth factors from the bone matrix that support tumour cell growth. In the aforementioned experiments, IL-6 deficient cells grew slower in the bone microenvironment than their non-target counterparts, and it is highly likely that this is due to the lack of IL-6 action within the vicious cycle. This assumption is supported by the fact that no growth retardation was observed in tumours derived from IL-6 deficient cells implanted into the mammary fat pad.

My findings may have clinical implications since the anti-human IL-6R antibody, Tocilizumab, is clinically available and therefore could be used in the treatment of patients with advanced and intractable metastatic IL-6 producing (oestrogen receptor negative) breast cancer.

Keywords:

Intereukin-6, breast cancer, metastasis, vicious cycle

Zusammenfassung

Brustkrebs metastasiert vorwiegend in den Knochen, wo er zu mehrheitlich osteolytischen Läsionen führt. Interleukin-6 (IL-6) spielt eine wichtige Rolle in der Reaktion des Körpers auf Verletzungen und bei Infektionen. Es ist weiterhin an der Immunantwort, Entzündungsvorgängen und der Hämatopoese beteiligt. Eine Dysregulation des IL-6 Haushaltes spielt bei vielen Krankheiten und auch bei Brustkrebs eine wichtige Rolle. Es gibt viele klinische Hinweise darauf, dass hohe IL-6 Titer im Blut bei Brustkrebspatientinnen mit einer schlechten Prognose und schneller Krankheitsprogression einhergehen. Umgekehrt haben mehrere *in vivo* und *in vitro* Studien gezeigt, dass niedrige IL-6 Level mit einem verringerten Krebszellwachstum einhergehen. Daher scheint IL-6 eine wichtige Rolle bei der Entwicklung von Brustkrebsmetastasen im Knochen zu spielen.

Das Hauptanliegen dieser Arbeit war es, die Rolle von IL-6 bei der Brustkrebsmetastasierung und dem Brustkrebswachstum im Knochen besser zu verstehen. Um dieses Ziel zu erreichen, wurde ein Xenotransplantatmodell osteolytischer Brustkrebsmetastasen verwendet, bei dem die IL-6 Produktion durch die Brustkrebszelle durch genetische Manipulation reduziert wurde, beziehungsweise der IL-6 Rezeptorsignalweg durch einen humanisierten IL-6 Rezeptorantikörper blockiert wurde. Folgende spezifische Ziele wurden definiert:

1) Beurteilung der Auswirkungen des IL-6 Knockdowns in humanen MDA-MB-231 Brustkrebszellen auf das Krebszellwachstum im Knochenmilieu *in vivo* und *in vitro*.

2) Beurteilung der Auswirkungen des IL-6 Knockdowns auf das Brustkrebszellwachstum außerhalb des Knochenmilieus (im mammären Fettpolster).

3) Beurteilung des autokrinen Effektes der IL-6 Signalübertragung auf das Wachstum von MDA-MB-231 Brustkrebszellen, unter Verwendung des IL-6 Rezeptor Antikörpers Tocilizumab.

Folgende Ergebnisse wurden erzielt:

1) Der Knockdown von Interleukin-6 (IL-6) in MDA-MB-231 Brustkrebszellen durch lentivirale Transduktion führte zu einer Senkung der IL-6 mRNA und der Proteinproduktion um mehr als 50 %. Dies hatte keinen Einfluss auf das Wachstum der MDA-MB-231 Zellen *in vitro*, senkte jedoch die invasiven Eigenschaften der untersuchten Zellen.

2) Im Vergleich zu den Non-Target Kontrollzellen führte der Knockdown der endogenen IL-6 Produktion durch stabile shRNA Expression zu einer signifikanten Verlangsamung des Zellwachstums im Knochenmilieu.

3) IL-6 Knockdown Zellen wuchsen genauso schnell außerhalb des Knochenmilieus wie die Non-Target Kontrollzellen, was darauf hindeutet, dass der Effekt von IL-6 auf das Krebszellwachstum durch das Knochenmilieu vermittelt wird.

4) Teilweise Inhibition des autokrinen IL-6 Signaltransduktionsweges durch Administration des humanisierten IL-6 Rezeptorantikörpers Tocilizumab in geringer Dosierung, führte zu einer sichtbaren, jedoch nicht statistisch signifikanten Wachstumsverminderung des Brustkrebszellwachstums im Knochenmilieu.

Wenn metastasierte Brustkrebszellen in das Knochenmilieu eindringen, stören sie den sorgfältig balancierten Knochenstoffwechsel und verschieben ihn meist zugunsten des Knochenabbaus. Der „Vicious Cycle“ ist ein Modell der Brustkrebsmetastasierung in das Skelett, welches erklärt, wie Brustkrebszellen den Knochenmatrixabbau durch die Aktivierung von Osteoklasten und Osteoblasten induzieren. Beim Abbau der Matrix kommt es zur Freisetzung von Wachstumsfaktoren aus dem Knochen, welche wiederum das Brustkrebszellwachstum fördern. In den oben genannten Experimenten wurde festgestellt, dass IL-6 defiziente Zellen im Knochenmilieu langsamer wuchsen als ihre Non-Target Kontrollzellen und es ist sehr wahrscheinlich, dass dies der fehlenden Wirkung des IL-6 auf den „Vicious Cycle“ geschuldet ist. Diese Annahme wird durch die Tatsache gestützt, dass keine Wachstumsverlangsamung des Tumors aus IL-6 defizienten Zellen im mammären Fettpolster, also außerhalb des Knochenmilieus, beobachtet werden konnte.

Die Ergebnisse könnten von klinischer Bedeutung sein, da der IL-6 Rezeptorantikörper Tocilizumab klinisch zur Verfügung steht und somit bei Patientinnen mit fortgeschrittenem, therapierefraktärem, IL-6 produzierendem (Östrogenrezeptor negativen) Brustkrebs zum Einsatz kommen könnte.

Schlüsselwörter:

Interleukin-6, Brustkrebs, Metastasierung, Vicious Cycle

ABBREVIATIONS

BMP	Bone morphogenetic protein
cDNA	Complementary DNA
DMEM	Dulbecco's Modified Eagle medium
DNA	Deoxyribonucleic Acid
ET-1	Endothelin 1
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GAPDH	Glycerylaldehyde-3-phosphate dehydrogenase
IGF	Insulin like growth factor
IL	Interleukin
JAK	Janus kinase
M-CSF	Macrophage colony stimulating factor
MMP-9	Matrix metalloproteinase
MSC	Mesenchymal stem cell
NFκB	Nuclear factor kappa B
OPG	Osteoprotegerin
PBS	Phosphate-buffered saline
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear kappa B
RANKL	Receptor activator of nuclear kappa B ligand
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase chain reaction
STAT	Signal transducer and activator of transcription
TIMP-3	Tissue inhibitor of matrix-metalloproteinase
TGF-β	Transforming growth factor beta
TNF	Tumour necrosis factor
TRAcP	Tartrate resistant phosphatase
VEGF	Vascular endothelial growth factor
μ-CT	Micro-computerised tomography

LIST OF FIGURES

1 INTRODUCTION

The human skeleton undergoes continuous life-long renewal. Old or damaged bone is removed by osteoclasts, multinucleated cells of hematopoietic origin, and replaced with new bone by bone-forming osteoblastic cells of mesenchymal origin. Cancer metastasis to the skeleton can greatly disturb this meticulously balanced process, leading to an imbalance between bone resorption and formation.

One of the key participants in physiological and pathological bone remodeling is the osteoblast, which is derived from mesenchymal stem cells. It synthesizes new bone matrix, comprised primarily of collagen and non-collagenous proteins, and also aids in the mineralization of the bone matrix. Upon stimulation by bone morphogenetic proteins and local growth factors, the mesenchymal stem cells proliferate and form pre-osteoblasts, which subsequently differentiate into mature osteoblasts. After synthesizing new bone, the osteoblasts either undergo apoptosis or become embedded in the bone as osteocytes. These cells have long processes that communicate with other osteocytes and with osteoblasts on the bone surface. The processes connect the entire matrix through a series of canaliculi (1). The osteoblast plays a pivotal role in osteoclastogenesis and osteoclast activation as it synthesizes RANKL (2) (see below).

Another key participant is the multinucleated osteoclast (3) whose major function is to degrade bone matrix. It develops from a bone-marrow derived population of mononuclear precursor cells circulating in the monocyte fraction of the peripheral blood (4). On the bone surface, osteoclast precursors differentiate into fully functional bone-resorbing cells. This process requires the presence of macrophage colony stimulating factor (M-CSF) and osteoblast-derived receptor activator of nuclear Factor κ B ligand (RANKL), a member of the TNFα superfamily (5). RANKL interacts with its cognate receptor on the osteoclast surface, receptor activator of nuclear factor κ B (RANK).

Both M-CSF and RANKL are necessary and sufficient factors for osteoclastogenesis (6). Apart from stimulating osteoclast formation and activation, RANKL also increases osteoclast survival and adherence (7).

1.1 Pathophysiology of bone metastasis

1.1.1 Cancer and bone metastasis

In 1889 Stephen Paget suggested that the development of bone metastasis is not a random process but based on what he termed the “seed and soil” theory. This theory took into account the qualities of both the tumour and the host tissue. He noticed that some tissues provide a more fertile “soil” for cancer metastasis than others and that bone was among the preferred metastatic sites for tumours.

Bone provides an especially favourable environment for the aggressive behaviour of metastatic cells (8). In particular, breast, prostate, lung, renal-cell and colorectal cancers have the propensity to metastasize to bone, altering bone homeostasis and rendering the disease systemic and essentially incurable (8)–(9). Even though bisphosphonates provide an effective treatment that can prolong survival and improve quality of life, currently there is no treatment that can cure bone metastatic disease (10).

The occurrence of bone metastasis affects both the quality of life and the life expectancy of tumour patients. While occult and symptomless at the early stage of development, bone metastases can lead to significant morbidity at later stages. The most severe symptoms include intractable pain, pathological fractures, leukoerythroblastic anaemia (11), neurologic symptoms (e.g. spinal cord compression) and hypercalcemia (8).

Bone metastases are thought to form as follows: Firstly, the primary tumour cells invade their surrounding tissue secreting proteolytic enzymes and then enter the circulation. Even though many solid tumours preferentially metastasize to the bone, distant site tumour metastasis is considered a rather inefficient mechanism. Animal models showed that only 0.01% or fewer of the cancer cells entering the circulation develop into metastases (12). The cells that do survive have to overcome the wall of the bone marrow sinoids, invade the marrow stroma and generate their own blood supply.

1.1.2 Breast cancer and bone metastasis

Breast cancers can be oestrogen receptor negative [ER(-)] or positive [ER(+)], with ER(-) cancers showing a more aggressive metastatic phenotype. At advanced stages, breast cancers metastasize to bone in 70% of cases. Metastases of both phenotypes of breast cancer lead to increased bone turnover as determined by urine and serum bone remodelling markers (7).

Breast cancer cells, however, are unable to degrade bone directly. To achieve degradation of bone matrix they need to recruit and stimulate osteoblasts to produce osteoclast-stimulating factors which then lead to osteoclast activation and bone degradation (5). Current therapies, such as bisphosphonates (synthetic analogues of inorganic pyrophosphates), are directed at blocking osteoclast activity and slow down lesion formation. Although they reduce skeletal-related events, they do not lead to restoration of the bone, which is probably due to the fact that the osteoblasts are functionally “paralysed” by breast cancer cells. In osteoblasts breast cancer cells lead to increased apoptosis, a change in morphology and suppression in differentiation and mineralization, evidenced by a lack of expression of alkaline phosphatise, bone sialoprotein, and osteocalcin (13).

The abundance of RANKL in the bone microenvironment might be one of the so called “soil”- factors for RANK expressing breast cancer cells. The ER(-) human breast cancer cell line MDA-MB-231 expresses the RANK on its surface and RANKL induces the migration of these cells that can be blocked by osteoprotegerin (OPG). This theory is likely to be associated with the choice of RANK expressing cancer cells for bone as their preferential metastatic site (14) (15). This is, however, not fully proven.

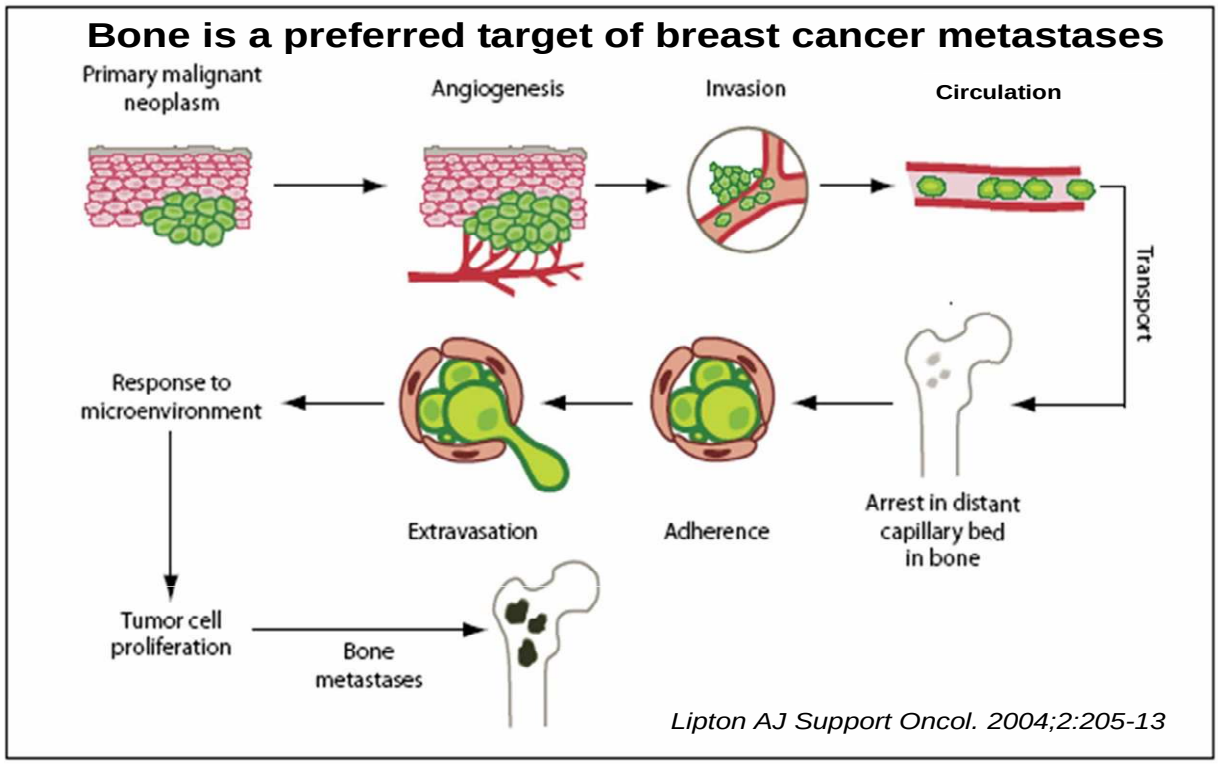


Fig. 1: **Model of cancer metastasis**
A simplified model depicting cancer metastasis to bone but omitting most of the factors mentioned in the text above. The malignant cells multiply in the bone microenvironment. As the tumour grows its oxygen and nutrient requirements increase and stimulate secretion of angiogenic factors that result in new vessel formation to ensure blood supply. The tumour grows and is able to send off metastatic cells through those newly formed vessels into the circulation. The metastatic cells get arrested in the bone tissue where they adhere to the capillary walls. After leaving the capillaries they interact with the microenvironment of the host tissues and proliferate.

1.1.3 Types of bone metastasis

Based on clinical and radiological terms, the lesions caused by bone metastases are usually divided into two types, osteolytic or osteoblastic. Osteolytic lesions occur through degradation of bone, whereas osteoblastic lesions are characterized by excess bone formation. The newly formed bone, however, is of inferior quality with a weak and disorganized structure which leads to pathological fractures (11). However, most bone metastases contain both osteoblastic and osteolytic elements, with one being more prevalent than the other.

In breast cancer bone metastases, osteolytic lesions are more common. In these cases, osteoclast activity is increased whereas osteoblast activity is impaired (8). Almost always, these osteolytic lesions contain an osteoblastic component which is regarded as a futile attempt at bone repair by the osteoblast and explains the elevated levels of alkaline phosphatase in patients with osteolytic bone metastasis (11).

1.1.4 Breast cancer bone metastasis and the vicious cycle

The model that to date gives the best explanation why tumour cells metastasize preferentially to bone and thrive in the bone environment was conceptualized by a pioneer in the tumour-bone microenvironment field, Dr. Gregory Mundy and termed the "vicious cycle" (11) (16).

Breast cancer cells are incapable of directly degrading bone or activating osteoclasts (5). Instead, they indirectly cause lytic bone lesions by producing factors such as parathyroid hormone related protein (PTHrP), tumour necrosis factor α (TNF-α) and interleukins 1, 6, 8 and 11 (17). These factors, especially PTHrP, stimulate osteoblast expression of RANKL which stimulates osteoclast differentiation and activation via interaction with its corresponding receptor RANK on the osteoclast surface. Osteoclasts then resorb bone, leading to the release of growth factors from the bone matrix such as bone morphogenic protein (BMP), insulin like growth factor-1 (IGF-1) and transforming growth factor β (TGF-β), etc. The release of these growth factors supports cancer cell proliferation and induces further secretion of PHTrP from the cancer cells (12).

Yin *et al.* created a TGF-β unresponsive MDA-MB-231 cell line which showed significantly slower progression of osteolytic bone lesions than parental cells of the same lineage, proving that TGF-β is a growth factor for tumour cells. On the other hand, TGF-β receptor over-expressing MDA-MB- 231 cells showed increased tumour burden and osteolytic lesions (18).

Degradation of the bone matrix not only leads to the release of growth factors from the matrix but also of calcium. Tissue calcium levels around lytic lesions can reach concentrations of up to 40 mM, compared to serum calcium concentrations of 2.5mmol/L. It was suggested that these high concentrations stimulate PTHrP expression by MDA-MB-231 cells (19).

Moreover, metastatic breast cancer cells induce a loss of function in osteoblasts. They suppress osteoblast differentiation, alter their differentiation and increase their apoptosis. When cocultured with breast cancer cells osteoblasts produce higher levels of IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1), factors that indicate an osteoblast inflammatory response (13).

1.1.5 Increased bone turnover promotes breast cancer cell growth in bone

Zheng *et al*. demonstrated that accelerated bone resorption promotes breast cancer cells growth in bone (20). Balb/c nu/nu mice receiving a low calcium diet for 3 days prior to tumour inoculation developed secondary hyperparathyroidism and increased bone remodelling. The latter was proven by increased osteoclast and osteoblast numbers, as well as higher circulating levels of tartrate resistant acid

phosphatase 5b (TRAcP5b) and osteocalcin. When MDA-MB-231 breast cancer cells were injected into the tibiae of these calcium-deficient animals, tumours grew faster as compared to mice on a normal calcium diet (20).

1.2 Interleukin-6 in cancer and bone metastasis

1.2.1 Interleukin-6

IL-6 is a pleiotropic cytokine originally identified as a B-cell differentiation factor. It is, however, also involved in various other physiological and pathological processes in the body. IL-6 is produced by macrophages, B-cells, T-cells, endothelial cells, osteoblasts (13) and tumour cells (21) and is active in the immune response, haematopoiesis, the acute phase response and inflammation. It contributes to the physiological function of the brain, heart and vessels, immune system and liver. Pathological conditions with increased IL-6 levels include autoimmune diseases, osteoporosis, and various cancers (22)–(23). The extensive implications of IL-6 in numerous diseases make it an important therapeutic target.

IL-6 is a 25 kDa protein (24) with a helical structure (23). It belongs to a family of cytokines all of which transduce signals through the gp130 protein. The family consists of 10 cytokines including IL-6 itself, interleukin 11 (IL-11), ciliary neurotropic factor (CNF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), leukemia inhibitory factor (LIF), neuropoietin (NPN), oncostatin M (OSM) and the recently discovered interleukins 27 and 31 (IL-27, IL-31). The effects of all of these cytokines are mediated trough the gp130 subunit of the IL-6 receptor family and 2 different pathways, either the mitogen-activated protein kinase (MAPK) or the signal transducers and activators of transcription (STAT) pathway. The activation of either depends on the ligand that interacts with the IL-6R and is tissue specific (25).

1.2.2 Interleukin-6 receptor

The IL-6R belongs to the class I cytokine receptor family which consists of high-affinity ligand-binding and signal-transducing components and is thus termed a multi-chained receptor complex.

The receptor complex consists of two molecules IL-6Rα and Glycoprotein 130 (gp130). IL-6Rα is an 80-kDa molecule consisting of 467 amino acids (24) and exists in a membrane-bound and soluble form which is generated by limited proteolysis of the membrane-bound protein and translation from alternatively spliced messenger RNA (26). The membrane-bound

IL-6R can be found on hepatocytes, neutrophils, monocytes/macrophages and some lymphocytes, whereas the soluble IL-6R has been found in numerous body fluids. A natural inhibitor of the sIL-6R and IL-6 complex is the soluble gp130, which does not inhibit the actions of the membrane bound IL-6R (27).

Gp130, the signal-transducing subpart of the receptor, is membrane bound and can be found on almost any cell (22). The soluble IL-6Rα (sIL-6R) and IL-6 complex acts as a proinflammatory mediator and binds to gp130 which makes any gp130 bearing cell susceptible to IL-6 (22). This process has been termed trans-signalling (26). After IL-6 binding, gp130 forms a homodimer and initiates intracellular signalling (27).

Gp130 can activate either the MAPK or STAT3 pathways. STAT3 is usually stored in the cytoplasm as an inactive monomer and forms homodimers after activation through phosphorylation trough a janus protein-tyrosine kinase (JAK). It then enters the nucleus and activates genes which in the case of breast cancer cells are growth and survival promoting (28)–(29). However, other groups report that the STAT3 pathway is anti-proliferative, apoptotic, osteoblastic and osteoclastic whereas the MAPK pathway is rather mitogenic, anti-apoptotic, anti-osteoblastic and anti-osteoclastic (30).

1.2.3 Biological functions of interleukin-6

The pleiotropic cytokine IL-6 was originally identified as the factor responsible for inducing immunoglobulin production in B-lymphocytes. Later on it has been implicated in numerous biological functions, wherein the effect on the immune system is considered most prominent (22).

In the immune system, IL-6 is involved in the proliferation and differentiation of T-lymphocytes into cytotoxic T cells and the normal development of B-cells. Moreover, it is known to stimulate hepatocytes to produce various acute phase proteins, for instance C-reactive protein (CRP), regulate fever (21, 22) and the release of anterior pituitary hormones comprising prolactin, growth hormone and luteinizing hormone.

IL-6 gene expression can be modulated by different inflammatory stimuli like HIV, Herpes viruses and a variety of cytokines like interleukin-1 and platelet derived growth factor (PDGF). Moreover, IL-6 is involved in stromal remodelling processes like wound healing through up-regulation of vascular endothelial growth factor (VEGF) and thus angiogenesis (21). The involvement in pro-angiogenetic processes is also believed responsible for the tumour-promoting properties of IL-6.

Its hematologic functions include the expansion of hematopoietic progenitors and proliferation and differentiation of megakaryocytic progenitors (31) (22). The cytokine and its receptor are expressed in neurons of hypothalamic nuclei that regulate body composition. IL-6 levels correlate with the body mass index. It was shown that IL-6 deficient mice developed mature-onset obesity (32).

IL-6 plays an important role in bone metabolism. It induces osteoclastogenesis and osteoclast activity that leads to increased bone resorption. Moreover, knock-out and transgenic mouse models show that IL-6 suppresses bone formation because IL-6 deficient mice show increased bone formation and IL-6 over-expressing mice show decreased osteoblast and osteoid numbers (30).

Liu *et al.* postulate that the bone protecting effect of estrogens and androgens is mediated through the inhibition of IL-6 expression (25). After menopause when oestrogen levels decrease and their bone-protective effect is abrogated, the IL-6 levels in the body increase. This effect is considered one of the main reasons why postmenopausal women develop osteoporosis (24).

Parathyroid hormone (PTH) is released from the parathyroid gland when serum calcium levels are low. In the bone it stimulates the release of RANKL from osteoblasts. RANKL in turn mediates the bone-resorptive PTH actions by activating osteoclasts through the receptor RANK. IL-6 may enhance this process by also increasing osteoblast expression of RANKL. In experiments on mice an IL-6R antibody was able to inhibit PTH induced bone resorption.

IL-6 fortifies the effect of prostaglandin E_2 on osteoblasts by increasing the expression of the prostaglandin receptors EP_2 and EP_4 on the osteoblast surface. This effect inhibits the OPG production in the osteoblast and thus increases RANKL/RANK interaction which in turn leads to increased osteoclastogenesis and bone resorption (25).

Other roles of IL-6 include the stimulation of the endometrial vasculature during the menstrual cycle, promotion of spermatogenesis, stimulation of epidermal proliferation, neural cell differentiation (24) as well as stimulation of fracture healing and bone mechanical resistance (30).

1.2.4 Pathological functions of Interleukin-6

IL-6 plays an important role in many autoimmune diseases like rheumatoid arthritis (RA), systemic onset juvenile idiopathic arthritis, systemic lupus erythematosus and inflammatory bowel disease (33). Diseases that are accompanied by long periods of inflammation lead to

cachexia and muscle loss. This process is mediated by IL-6 presumably through its enhancement of corticosteroid secretion (22).

With increasing age, the levels of IL-6 detected in the body increase because of decreasing levels of oestrogen and testosterone which normally down-regulate IL-6 secretion. This is believed responsible for changes that come with older age and resemble those of chronic inflammatory disease, like decreased lean body mass, low grade anaemia, lymphoproliferative disorders, decreased serum cholesterol and albumin, increased acute phase proteins and serum amyloid, osteopaenia and Alzheimer's disease (24).

For this work, however, the implication of IL-6 in the pathology of cancer is of particular interest, especially its implication in breast cancer and its metastasis to bone.

1.2.5 Interleukin-6 in cancer

Interleukin-6 is constantly expressed by renal, bladder, prostate, cervical, glioblastoma, colon and breast cancer cells. Breast, prostate, renal, myeloma and ovarian cancers also express the IL-6R (21).

In many cancers high levels of IL-6 are associated with a poor prognosis (34). Those cancers include multiple myeloma, lymphoma, ovarian cancer, prostate cancer, metastatic renal carcinoma and breast cancer (22). In multiple myeloma, prostate cancer and cholangiocarcinoma, IL-6 serves as a growth factor (28).

IL-6 supposedly promotes tumour growth through enhancing the production of anti-apoptotic and angiogenic proteins. For example, IL-6 stimulates the production of VEGF in tumour cells which in turn increases the endothelial cell proliferation and new vessel formation and thus tumour survival (35). Moreover, it could be shown that in oesophageal carcinoma and multiple myeloma cells IL-6 promotes survival though the induction of proteins such as b-cell lymphoma 2 and X1 (BCL-2 and BCL-X1) and induced myeloid leukaemia cell differentiation protein – 1 (MCL-1) that inhibit apoptosis (36).

IL-6 is held responsible for the occurrence of B-symptoms like night sweats, fever, and weight loss (24). Zaki *et al*. showed that it is specifically tumour secreted IL-6 that leads to tumour induced cachexia. When human prostate cancer bearing nude mice were treated with the IL-6 antibody CNTO 328 tumour cachexia was reduced relative to that in the untreated control group (32).

1.2.6 Interleukin-6 in breast cancer

High levels of IL-6 were significantly correlated to a negative prognosis and shorter survival in breast cancer patients (36), (28), (37) regardless of the ER-status of the tumour.

Median serum IL-6 levels are about 10 times higher (6.0 pg/ml serum) in patients with metastatic disease than in those with localized disease (38). The levels of IL-6 in patients with more than one metastatic site are significantly higher than serum IL-6 levels in patients with one or no metastatic sites (6.9 pg/ml serum) (39). One explanation for this was given by Salgado *et al*. who stated that circulating IL-6 in the serum may be partly derived from spill-over of tumour produced IL-6 and that thus high numbers of metastatic sites account for high IL-6 serum-levels in affected patients (21).

Progressive tumour disease is characterized by an unbalanced cell proliferation, acquisition of invasive and metastatic potential and evasion from the immune response (21). It could be shown that in patients with progressive disease IL-6 levels are higher (86.0 pg/ml) than in patients with non-progressive disease (40).

High levels of tumour-produced circulating IL-6 correlate with the number of metastatic sites (21) and IL-6 promotes the formation of distant metastasis (22). Different mechanisms for this have been suggested. For example IL-6 might be involved in the formation of distant metastasis in breast cancer by increasing the chemotactic motility of breast carcinoma cells. Furthermore, IL-6 may mediate local osteolysis via increasing osteoclast activity (41) (34).

1.2.7 Interleukin-6 in MDA-MB-231 (ER-receptor negative breast cancer) cells

In breast cancer, IL-6 has been associated with enhanced tumour cell growth, survival and immune evasion (28). Many human breast cancer cell lines are tumorigenic in nude rodent xenograft models, but the MDA-MB-231 cell line, developed by Toshiyuki Yoneda, is of particular interest as it is strongly ‘bone-seeking’ (42). This cell line is oestrogen receptor-negative, which gives it a more invasive profile with higher metastatic potential than its ER-positive counterpart, the MCF-7 cell line (43). Both cell lines possess the IL-6R and are thus both susceptible to IL-6 effects. The ER(-) MDA cells, however, are also able to produce and secrete high levels of IL-6 (28). The increased IL-6 secretion by MDA-MB-231 cells is due to increased activation of STAT3 in those cells (29). The constant STAT3 activation in MDA-MB-231 cells is probably due to autocrine signalling events since coculture of MDA cells with IL-6-containing medium from marrow stromal cells does not lead to additional STAT3 phosphorylation and activation in those cells. In ER(+) cells, on the other hand, adding IL-6-

containing medium led to increased STAT3 activation (28). MDA-MB-231 cells express RANK-receptor and a TNFα-receptor (43). The proinflammatory cytokines TNFα, TGFβ and IL-1β reportedly upregulate IL-6 production in MDA-MB-231 cells (41).

1.2.8 Interleukin-6 and breast cancer bone metastasis

Osteolytic breast cancer metastases also contain inflammatory cells such as macrophages and fibroblasts, which are considered to enhance tumour growth, invasion and metastasis. Infiltration of monocytes correlates with poor prognosis of breast cancers. Monocytes are recruited by cytokines and chemokines such as macrophage colony stimulating factor (M-CSF) and monocyte chemo-attractant protein (MCP-1) which are highly expressed by breast tumour cells. In the tumour microenvironment, monocytes undergo activation and differentiation and are designated tumour-educated or tumour-associated macrophages (TAMs) (44).

Lau *et al*. found that one mechanism through which tumours control their osteolytic activity is induction of osteoclast differentiation from tumour-associated macrophages. This formation is supported by RANKL and M-CSF produced by cells like fibroblasts and osteoblasts. They state that breast cancer cells do not only stimulate RANKL-induced osteoclast formation but produce a soluble factor that could be responsible for the RANKL-independent transition from a mononuclear phagocyte to an osteoclast (45).

According to Kudo *et al*. this soluble factor could be IL-6, as they showed that IL-6 induces osteoclast differentiation by a RANKL independent mechanism. If this is true, MDA cells induce bone resorption through the vicious cycle and through IL-6 induced osteoclast differentiation and thus enhance PTHrP induced hypercalcemia which forms an independent mechanism but enhances the vicious cycle (46). The question how important that mechanism is compared to RANKL induced osteoclast formation remains to be answered.

Selander *et al*. found that blocking the IL-6R signalling in MDA-MB-231 cells reduces the malignant potential of these cells (47). Whether this is due to the reduced endogenous IL-6 production in those cells or whether other factors under the control of the IL-6R are responsible for their decreased malignancy remains unknown. Breast cancer cells significantly increase the IL-6 production in osteoblasts which leads to increased osteoclast activation and bone resorption. The concomitant decrease in collagen and osteocalcin secreted by osteoblasts co-cultured with breast cancer cells confirms that breast cancer cells suppress osteoblast function in a manner consistent with inflammation-induced bone loss observed in bone pathologies (13, 48).

1.3 Animal models in breast cancer research

1.3.1 Models of metastasis in breast cancer

Animal models of bone metastasis investigating the behaviour of human cancers should meet the following requirements: Firstly, the genetic and phenotypic changes that human cancers undergo during their development should be repeated. These changes include migration, invasion, angiogenesis within the bone microenvironment, survival and growth with ensuing modifications through the environment. Secondly, the models have to reproducible and progress as quickly as possible to allow timely interventions and measurements.

Animal models are important to investigate the process of bone metastasis *in vivo* to fully understand the pathophysiology of the metastasis process and derive treatment opportunities for humans. Finding a perfect animal model to mimic the human setting is quite difficult because the pathogenesis of cancer in rodents and smaller mammals can differ quite significantly from that in humans. However, there are different animal models of bone metastasis that are able to duplicate certain selected aspects of human cancer metastasis and thus form an important part of the progress and advance of knowledge in the field of cancer biology. Further improvements and refinements of the animal models are being developed continuously (49).
Animal models of bone metastasis include:

1. Spontaneous tumours that occur in rodents or small mammals
2. Syngeneic transplantation of spontaneously arising rodent cancers
3. Chemical induction of cancers in selected strains of rats and mice
4. Transgenic mouse models
5. Rodent xenograft models of bone metastasis utilising immune incompetent animals

1.3.2 Nude rodent xenograft models of bone metastasis

Nude rodent (mice and rats) xenograft models are widely used by researchers because they can be rapidly reproduced and can represent one or more steps of the metastasis process depending on the field of interest. Nude (nu/nu) mice are genetically modified animals in such that they lack a thymus gland which causes immunodeficiency due to a lack of T lymphocytes and thus cell mediated immunity. The T cell lacking mice thus become tolerant to implanted tissues from other species including human cancer cells. The bone metabolism in these animals remains

unaltered and thus can be used as a model for the bone-tumour interactions that can also be seen in the human body.

To investigate the bone metastasis process the xenografted tissues or cells can be injected subcutaneously into the left ventricle of the heart. To investigate the ability of cancer cells to grow in bone, or to study cancer cell interactions with bone microenvironment, cancer cells can be implanted directly into the tibia or femur. To investigate cancer cell behaviour in other metastasis sites, cancer cells can be implanted subcutaneously into the mammary fat pad, the prostate gland or the lungs (49).

In breast cancer, many human derived cell lines cause tumours in nude rodent xenograft models. They are usually established either by intracardiac, intratibial or intra-mammary injection of these cells into the immunocompromised animals (49). These methods lead to the development of tumours including bone metastasis. The choice of the appropriate model depends on the step of the metastasis process that is under investigation.

Direct delivery of tumour cells to bone: To create a model of tumour metastasis to the bone environment, tumour cells can be injected directly through the tibial cortex of nude mice, into the bone marrow space below the proximal tibial metaphysis. These tumour cells establish themselves well in the environment and produce lytic lesions at the injection site (50).The advantage of this model is the relatively reliable creation of a tumour in a predetermined site with a known number of cells, while the contralateral tibia can be used as a control. This model allows the evaluation of the effects the tumour has on the bone microenvironment while the steps of extravasation and establishment of micrometastasis are bypassed. The disadvantages of this approach are that the piercing of the cortex and displacement of bone marrow in themselves produce a marked inflammatory response that influences the local bone metabolism and might significantly alter the bone microenvironment.

This model has been used in all experiments of this work concerning the bone microenvironment and tumour metastasis establishment in the latter, as the major aim of this project was to further investigate the factors that play a role therein.

1.3.3 Assessment of bone metastasis in nude rodent xenograft models

End-stage lesions of bone metastasis should be easily identifiable and reveal to which extent the tumour proliferation modifies bone structure so that conclusions that can be applied to humans in a clinical setting can be drawn. Overt or apparent lesions can be visualized by means of radiology in the living animals or histopathology after the animal is sacrificed and the tissue

harvested. A limitation to the x-ray method is that only severe lytic lesions and damage to the bone can be detected, whereas minor lesions and osteosclerotic lesions remain invisible. The new μ-CT technology can differentiate between osteolytic and osteosclerotic lesions regardless of the size.

To visualize micrometastasis more sensitive techniques like polymerase chain reaction (PCR) can be used to detect even very low amounts of tumour cells that get arrested in the capillary beds and have the propensity to develop into metastases. This might, however, lead to the overestimation of results (49).

It is thus not only important to pick the right animal model to investigate the step of the process in question but also to choose the right detection techniques and to interpret the results in the right way.

1.4 Hypothesis and aims

The role of IL-6 in breast cancer cell growth is controversial. In the literature, IL-6 is often considered a “double-edged sword” with some reports suggesting that IL-6 has tumour promoting properties, while others indicate a tumour inhibiting role. Despite these controversial findings, there is increasing evidence that high serum IL-6 levels are a negative prognosticator in breast cancer patients (36).

According to the model of the vicious cycle (see above), osteolytic cancer lesions in bone strongly rely on accelerated bone resorption, i.e. osteoclast activation through increased RANK/ RANKL interaction (11). The role of IL-6 within this process remains to be investigated. In the past, a number of studies have investigated the effect of IL-6 on individual components of the vicious cycle. IL-6 is known to stimulate bone resorption through an indirect mechanism, namely by activating the osteoblast to express RANKL. This in turn interacts with its cognate receptor, RANK, which is expressed by osteoclasts and stimulates these to actively resorb bone (17). Moreover IL-6 induces PTHrP production in osteoblasts in a non-cancerous setting (51), which in turn fosters tumour cell growth (13).

Furthermore, Kudo *et al*. report direct activation of osteoclasts through IL-6, while Zheng and colleagues found that fuelling the vicious cycle by a low calcium diet in human breast cancer bearing nude mice leads to an up-regulation in IL-6 mRNA in bone tissue (52).

Based on these results, I hypothesized that IL-6 plays an important role in breast cancer cell growth within the bone microenvironment.

The aim of my project was to determine the role of IL-6 in breast cancer cell growth in bone. To achieve this, I chose the IL-6 secreting MDA-MB-231 cell line (which is known to be highly malignant and bone seeking) and decided to knock down IL-6 production in these cells via shRNA technique. These IL-6 deficient cells were then characterized *in vitro* to determine whether IL-6 knock down had any effect on their growth and invasiveness.

In further *in vivo* studies, I then determined whether knock down of IL−6 in MDA cells reduces tumour growth in bone and non-bone tissues *in vivo*. Lastly, the human breast cancer cells were introduced into a murine environment. While murine IL-6 does not interact with the human IL-6R on the tumour cell surface, human IL-6 is able to interact with the murine receptor. This means that the IL-6 that is produced by the tumour cells will be able to exert its effects on the murine bone microenvironment but not the other way round (28). To further test whether IL−6R antibodies targeting tumour derived IL−6 may have any effect on tumour growth, the humanized antibody Tozilizumab was used to inhibit autocrine and paracrine stimulation of MDA-MB-231 cells in the murine bone microenvironment.

2 MATERIALS AND METHODS

2.1 Tissue culture

2.1.1 Breast cancer cell line

The human oestrogen receptor-α negative cell line MDA-MB-231 was obtained from Dr. T Yoneda (M.D. Anderson Cancer Care Center, San Antonio, TX, USA). All tissue culture media and supplements were obtained from Invitrogen (Carlsbad, CA, USA).

2.1.2 Cell cultures

The cells were cultured in T175 and T75 cm² plastic tissue culture flasks in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS, JRH Biosciences, KS, USA) and 1% penicillin-streptomycin antibiotic solution. The cultures were maintained at 37° in a humidified atmosphere of 95% air and 5% carbon dioxide (CO_2) and subcultured every third day when they reached about 80% confluence.

For subculture, the media and phosphate-buffered saline solution (PBS) and trypsin were incubated in a water bath until they reached a temperature of 37°C. The old media was removed from the flasks using a pump and the remainders were washed away with PBS to avoid the inactivation of trypsin by media remnants. 1mL of trypsin was then added and the cells were washed gently before 2mL of trypsin were administered for a T75cm² flask or 4ml for a T175cm² flask and left to incubate at 37°C for 5 minutes.

To ensure that all cells were lifted properly, the flasks were tapped gently and assessed under the microscope. To terminate the trypsin activity medium containing 10% FCS was added and the cell suspension was transferred to a 50mL Falcon tube. The cells were then separated by a syringe with a 21-gauge needle, counted using a haemocytometer and the cell viability was assessed by trypan blue exclusion. The mechanism behind this exclusion test is such that dead cells have damaged cell membranes which allow the trypan blue dye to penetrate the cell whereas live cells are not stained. This is a standard procedure in tissue culture to determine the number of viable cells in a cell suspension. Based on the cell count (to maintain the same amount of cells in all flasks) an appropriate dilution was calculated for the propagation of cells in the new flasks. The flasks were then incubated as before at 37°C, 5% CO_2 and 100% humidity. Media were changed every 2-3 days until they reached 80% confluence.

2.2 *In vitro* experiments

2.2.1 Knock down of IL-6 production in MDA-MB 231 using the shRNA technique

The knockdown of IL–6 in MDA-MB 231 cells was performed with my supervisor Yu Zheng. Briefly, a lentivirus vector-based shRNA expression system (Sigma, USA) was used to knock down endogenous IL-6 mRNA via shRNA in MDA-MB 231 cells.

The IL-6 mRNA consists of 1.2-1.3 kilo base pairs containing 0.6 kb of coding region and approximately 0.5 kb of non-coding region followed by a poly A tail (Cytokines in animal health and disease by Michael J. Myers). Three constructs were identified that targeted the protein translation region of the IL-6 mRNA at 3 different positions, i.e. positions 211, 272 and 636.

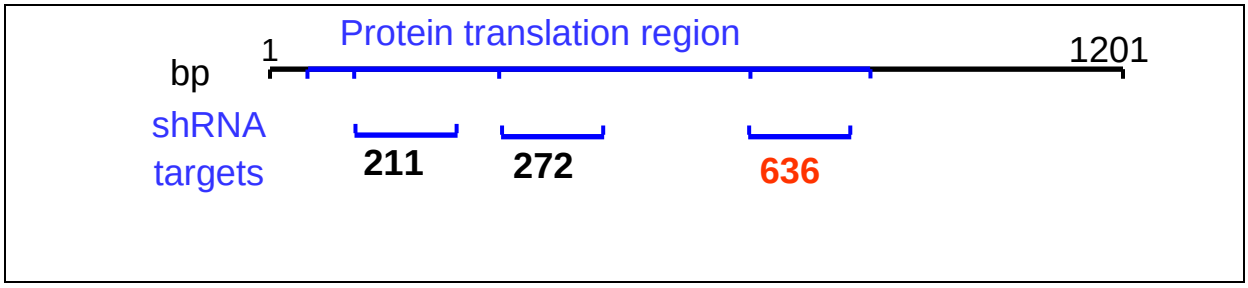


Fig. 2: **IL-6 mRNA sequence**
Shows the sequence of the IL-6 mRNA which is 1201 bp long. 3 constructs that matched the mRNA at positions 211, 272 and 636 were tried. The best results were obtained by the 636 construct which was hence used for the knockdown and is the reason why the knockdown cells were called MDA-636

The target regions at bp sequence numbers 211, 272 and 636 of the protein translation region of the IL-6 mRNA were targeted. Targeting the protein translation region at position 636 gave the best knockdown results, so for all further experiments only the 636 shRNA were used. The control cells were transfected with a lentivirus vector encoding a non–target sequence. The success of the transfection process in both knockdown and non-target cells was ensured by puromycin selection (4 μg/mL) for two weeks. Fig. 2 shows the IL-6 mRNA sequence with its protein translation region.

2.2.2 RNA extraction

Ribonucleic acid (RNA) extraction was performed by means of an extraction kit (innuPREP RNA mini kit, Analytik Jena, Jena, Germany) following the manufacturer's protocol.

The cells were lysed by incubation in a lysis buffer which contains RNase inactivating chaotopic ions. Then the RNA and some of the deoxyribonucleic acid (DNA) were bound to the silica membrane provided. The DNA, salts metabolites and macromolecular cellular components were removed in several washing steps using washing buffers and the pure RNA was finally eluted under low ionic strength conditions with RNAase-free water. The RNA purity and integrity was checked by spectrometry (ration of 260/280 > 1.8) as well as through gel electrophoresis in a 1% agarose gel. RNA was routinely kept at -70°C for long-term storage to ensure stability.

2.2.3 Reverse transcription

Reverse transcription (RT reaction) is a technique that transcribes the RNA template into a complementary single stranded DNA (cDNA) molecule. To initiate the reaction the 11µL RNA were incubated with a reverse transcriptase enzyme a primer [(oligo (dT)] at 65°C for 5 minutes to denature the RNA secondary structure and anneal the primer. The samples were then chilled on ice quickly to prevent the reformation of the secondary structure. The RT reaction was then initiated by adding dNTPs, an RNAase inhibitor and the reverse transcriptase enzyme and incubated at 50°C for 45min-1h. The reaction was then terminated by heating the mixture to 70°C for 15 min.

2.2.4 Real time PCR

Real time PCR is a quantitative PCR method for the determination of cope number of PCR templates such as DNA or cDNA in a PCR reaction. We used the intercalator based RT-PCR method. A special thermocycler equipped with a sensitive camera that monitors fluorescence in each well of the 96-well plate that contains the samples at frequent intervals during the PCR reaction is needed. The intercalator-based method, also known as the SYBR green method, requires a double-stranded DNA dye in the PCR reaction which binds newly synthesized double-stranded DNA and gives fluorescence. We used SYBR Green with the iQ5 cycler (BioRad, Munich, Germany) and SYBR green dye (SYBR Green PCR Master Mix; Applied Biosystems, Foster City, CA, USA).

2.2.5 Measurement of IL-6 protein levels by means of IL-6 ELISA kit

The IL-6 protein levels were measured in the conditioned medium of parental MDA-MB-231 cells, non-target cells and knock down cells by means of an ELISA kit (Quantikine Human IL-6

immunoassay, BD). The 3 different cell types were seeded in triplicates into a 96 well plate at a concentration of $5x10^3$ cells per well. The conditioned medium was collected after 48 hours and the ELISA was performed following the manufacturer's protocol. The experiment was repeated 3 times.

2.3 Characterization of cells *in vitro*

2.3.1 Growth curve

To assess the proliferation rate, the parental, non-target and knockdown cells were cultured in Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% FCS and seeded at a concentration of 10^4 cells per well (3.8 cm^2) in twelve-well tissue culture plates and allowed to adhere overnight. The cells were then counted daily by Trypan blue exclusion after trypsinising until they reached 100% confluence on day four. The experiments were performed independently 3 times to ensure the validity of the results.

2.3.2 Chemoinvasion assay

The ER-negative MDA-MB-231 cell line is highly invasive and metastatic. The BD Biosciences Biocoat Matrigel Invasion Chamber kit (Fig. 3) was used to assess the invasive properties of the knock down versus those of the non-target cells that were used as controls.

The principle of this assay is to mimic the metastatic behaviour of malignant cells in the body, which unlike most benign cells, are capable of digesting the coated membrane (Matrigel) and crossing basement membranes and extracellular matrix. For this purpose the Engelbreth-Holm-Swarm sarcoma basement membrane "Matrigel" is commonly used.

The Matrigel inserts provided in the kit were brought to room temperature and rehydrated with 37°C warm serum-free DMEM in a humidified tissue culture incubator at 37°C and 5% CO_2 atmosphere. The medium was then carefully removed and the inserts were put into the wells of a 24 well companion plate which contained 0.75 ml of chemoattractant (5% foetal bovine serum in DMEM).

The cells were lifted from their flasks with trypsin which was inactivated with full serum medium after 5 minutes. To avoid the interference of the full serum medium with the experiment cells were centrifuged for 5 minutes at 1000rpm. The full serum medium was then removed and the cells were resuspended in serum-free medium. The knock down and non-target cells were seeded into the Matrigel covered and control (non Matrigel covered) inserts at a

concentration of $2.5x10^4$/insert. The plate was incubated in the humidified tissue culture incubator at 37°C and 5%CO_2 atmosphere for 22 hours.

After the completion of the incubation time the non-invading cells were removed by repeatedly (and quickly to avoid drying of the cells) “scrubbing” the membrane with a cotton swab inside the bottom of the membrane.

The non-invading cells on the surface of the Matrigel membrane were then removed with a cotton swab. The membrane was then detached from the transwell with a scalpel and the cells attached to the bottom of the membrane were fixated with formalin then stained with hematoxylin for 5 minutes.

After staining the membrane was put onto slides and the cells were counted after a drying period of 24h. The cells were counted in 3 representative areas in the center and the periphery of the membrane. The invading cells were counted and the invasion index calculated using the following formulas:

1. $$\%Invasion = \frac{MeanNumberOfCellsInvadingThroughMatrigelInsertMembrane \times 100}{MeanNumberOfCellsMigratingThroughControlInsertMembrane}$$

2. $$InvasionIndex = \frac{\%InvasionTestCells}{\%InvasionControlCells}$$

The Experiments were performed independently 2 times to ensure the validity of the results.

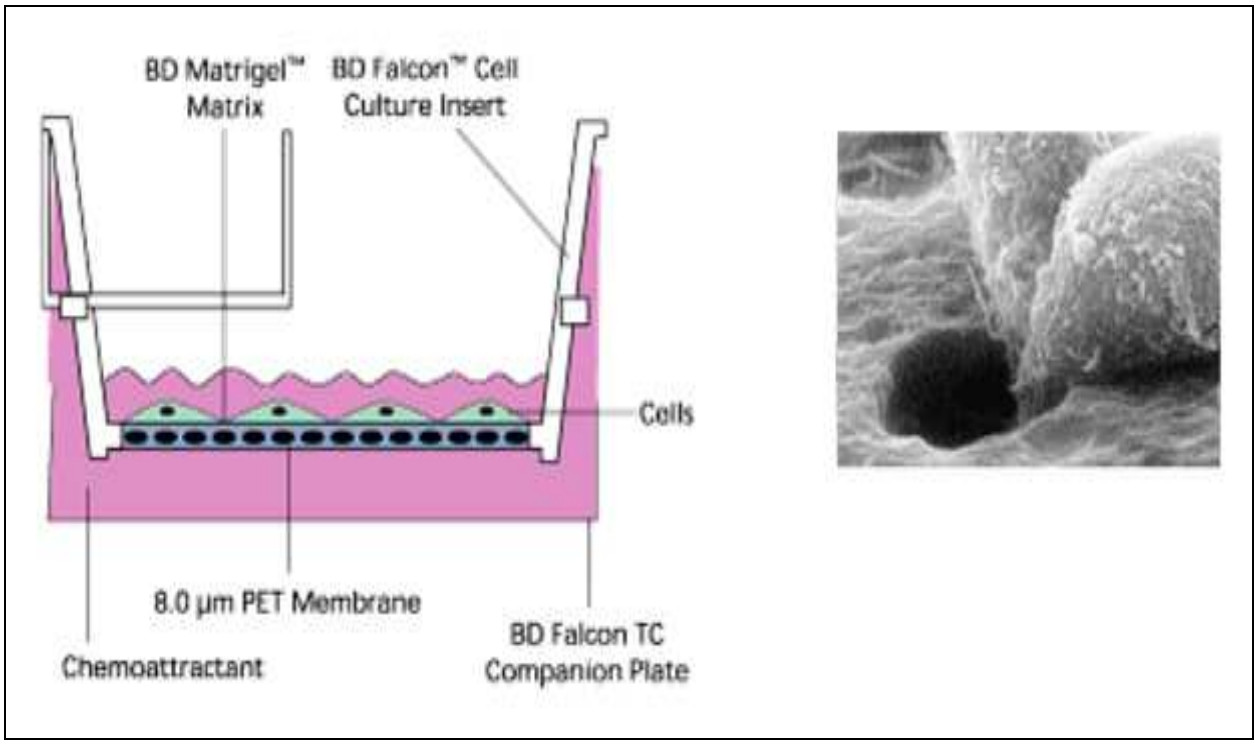


Fig. 3: **Chemoinvasion assay**
The picture on the right side shows a model of the BD Biosciences Biocoat Matrigel Invasion Chamber Mouse models of breast cancer growth. The invading cells are seated into serum-free medium on the bottom of the insert that is a porous membrane (pore size 8µm) covered by matrigel. The insert is put into a well containing 5%-foetal calf serum (FCS) medium that serves as a chemoattractant and incubated for 22h. The invading cells are attracted by the 5%-FCS medium in the well and use enzymes like matrix metalloproteinases to overcome the matrigel layer of the membrane and change the sides of the well. The image on the left hand side is a scanning electron micrograph provided by BD biosciences showing two human fibrosarcoma cells, having digested the BD Matrigel Matrix occluding the membrane and migrating through the 8 µm of the PET membrane.

2.3.3 Mouse maintenance

Four-week-old female BALB/c nu/nu mice (Animal Resources Centre, Canning Vale, WA, Australia) were used for the experiments. The mice were maintained under specific pathogen free conditions throughout the study at the Animal facilities of the ANZAC Research Institute in accordance with Institutional Animal Welfare Guidelines and an approved protocol and were allowed food and water ad libitum. All mouse manipulations were performed inside a laminar-flow hood under aseptic conditions whilst maintaining general anesthesia with intra-peritoneal injection of freshly prepared ketamine/xylazine (Sigma, St. Louis, MO, USA) at a dose of 75/10 mg/kg, unless otherwise noted. All anaesthetics were provided by Sigma-Aldrich. For all *in vivo* experiments, mice were monitored for changes in weight and behaviour and were euthanized by neck dislocation at the end point of the experiments.

2.3.4 Cell preparation for intratibial *in vivo* injection

The cells selected for *in vivo* injection were the non-target and knock down cells. Both cell variants were routinely passaged 1-2 times after revival from frozen stock before preparation for *in vivo* injection into nude mice. As before, all media and PBS were prewarmed. Instead of trypsin, Versene (0.02% EDTA) was used to lift the cells from the bottom of the flask as it is known do less damage to cells. After a PBS wash the cells were rinsed with Versene and then incubated in 5ml Versene (for 175 cm^2 flask) at 37°C for one hour with manual patting of the flask every 20 minutes to support cell detachment.

Following detachment from the flask, the Versene suspension was transferred into a 15 ml falcon tube and the cells were washed twice by centrifugation at 800g for 5 minutes in 10 ml PBS before an aliquot was taken to assess the viability of the cells by Trypan blue staining. Only suspensions with >97% viable cells were used for *in vivo* injections.

After a final PBS wash and centrifugation, the cells were resuspended in PBS at a concentration of 5 X 10^6/ml for both cell variants and injected into the tibiae of nude mice (5 X 10^4 cells in10μL each tibia). The cells were kept on ice to prolong survival until the end of the injection period.

2.3.5 Mammary fat pad injection

Matrigel was thawed on ice to prevent solidification for 2-3 hours prior to injection. 2x10^6 cells were grown per mouse (2 X 175 cm^2 to near 100% confluence for 10 mice). The cells were

detached from the flask bottom by Versene treatment, pooled into 50 ml Falcon tubes, centrifuged at 1000rpm for 5 minutes, washed with PBS and centrifuged again. The cells were then resuspended in 5 ml PBS, the cell concentration was determined by means of a haemocytometer and a volume of the suspension of 1.5 ml that contained $2x10^7$/ml cells was calculated. The required amount of the suspension was removed, spun down at 1000 rpm for 5 minutes and kept on ice for another 5 minutes while the Matrigel solution was prepared. The 1.5 ml Matrigel solution was prepared by adding 50% Matrigel into PBS. The cells were then resuspended in the Matrigel mix.

The Matrigel mix was put into 1ml syringes and put on ice to ensure the viability of the cells. The balb nu/nu mice were anaesthetized with 100-200µl ketamine/xylazine (depending on the size of the mouse) and 100µl cell suspension was injected at the flank into the 4th mammary fat pad visible under the skin of the nude mice. The mice were left in lying in the same position the one in which the injection took place to allow the Matrigel to solidify before the recovery of the animals.

The tumour was measured by digital callipers every 2nd day after injection for the duration of 16 days. On the 16th day the mice were euthanized under anaesthesia by neck dislocation.

2.3.6 Antibody treatment

The humanized IL-6R antibody Tocilizumab was administered every 3 days at a dose of 15mg/kg/3days via subcutaneous injection. The antibody was kindly provided by Prof. Frank Buttgereit, Charité Berlin.

The doses for the antibody treatments were based on a survey of existing literature where the antibodies were used for the treatment of rheumatoid arthritis (53-55). Tocilizumab was stored in aliquots in PBS at 4°C.

2.4 Anti-resorptive agents

To achieve rapid and profound inhibition of bone resorption by the highly resorptive MDA-MB-231 cells in the vicious cycle, recombinant Osteoprotegerin (OPG) was administered to the control groups of the knockdown cell and non-target cell injected mice. The reason for the use of OPG was to assess whether after shutting down the vicious cycle there would be a difference in the growth behaviour of the knockdown cell tumours as compared to the non-target tumours.

OPG consisting of amino acids 22-194 of human OPG fused to the Fc domain of human immunoglobulin G (Fc-OPG administered at a dose of 3mg/kg/3days) was kindly provided by Amgen Inc., Thousand Oaks, CA, USA. The dose of Fc-OPG is based on a literature survey.

The drug was stored in aliquots in acetate buffer, pH 5.0 at -80°C and was thawed and diluted in PBS immediately prior to use (56).

2.5 Radiologic methods

2.5.1 Faxitron X-Ray

The use of the Faxitron X-ray machine allowed *in vivo* monitoring of the developing bone lesions. The mice were anaesthetised as before and assessed by digital radiography (MX-50 X-ray cabinet, Faxitron, Wheeling, IL, USA) at 3 time points following injection. X-ray doses used were 26kV for 10 seconds for the long bones in anaesthetised mice (approximate magnification: 2 times). At the experimental endpoint the mice were anesthetised, examined radiologically for a last time, then sacrificed and the tissue harvested.

2.5.2 Micro-computerised tomography (µCT)

After tissue harvesting, representative microcomputed tomography images of tibiae were obtained using a Skyscan 1172 scanner (SkyScan, Kontich, Belgium). The scans were performed at 100kV, 100µA using a 1mm aluminium filter. In total, 1800 projections were collected at a resolution of 7.58µm per pixel. Reconstruction of the sections was performed using a modified Feldcamp cone-beam algorithm with beam hardening correction set to 50%. VGStudio MAX 1.2 software (Volume Graphics GmbH, Heidelberg, Germany) was used to obtain 3D visualisation of tibiae from reconstructed sections.

2.6 Tissue analysis

2.6.1 Tissue processing

The harvested tibiae were fixed in 4% paraformaldehyde buffered with 0.1 M PBS for 48h and decalcified using 10% EDTA at 4°C for 2 weeks with the EDTA solution changed every 3 days. The tissues were then processed and embedded in paraffin. Five-micron sections were cut from each specimen and stained with hematoxylin and eosin according to protocol for routine

histological examination. To facilitate the adhesion of the bone sections, the glass slides were pre-coated with 2% (3-aminopropyl) triethoxysilane in acetone solution and dried over night.

2.6.2 Measurement of lytic lesions

The lytic bone areas of cancer cell-injected tibiae were measured on digitally recorded radiographs using interactive image analysis software (ImageJ, NIH, USA) following careful identification and manual demarcation of lesion borders which were observed as radiolucent lesions in the hind limbs. The size of lesions was calculated and presented as X mm^2 at each time point. The 2-dimensional measurement of the lytic bone areas using X-ray analysis has its limitations and may not quite reflect the actual area/size of lytic lesions. However, the animal studies in this thesis were well controlled, the µCT images were used to verify the X-Ray measurement and this should overcome the inaccuracy caused by the measurement and produce reliable data.

ImageJ was also used for histological analysis of the harvested and processed tibiae. Measurements were performed in longitudinal 5µm sections stained with haematoxylin eosin. To determine tumour area, a representative sagittal sections was taken, that represented the upper mid and lower region of the proximal tibia. All tumour areas were measured including the surrounding soft tissues if the tumour broke into them, unless there was an independent tumour mass caused by leakage during the injection (which rarely occurred). Total tumour area was measured in each section and used as an index of tumour burden. Cortical bone area was measured in the same sections.

2.7 Statistical analysis

All data were represented as the mean ± SD (or SEM) and statistical analysis was performed using the one-way ANOVA, the two-way ANOVA and the t-test by means of PRISM 5 Graph pad programme (GraphPad Software La Jolla, CA USA). Significance was accepted where p was < 0.05.

3 RESULTS

3.1 *In vitro* experiments

3.1.1 Knock down of interleukin-6 production in MDA-MB-231 cells using the shRNA technique

To assess the role of IL-6 produced by MDA-MB-231 cells in their growth in the bone micro-environment we decided to knock down the production of this protein in this cell line using the shRNA technique. As mentioned above, the MDA-MB-231 cells were transduced with a viral vector containing the sequence for an IL-6 mRNA targeting shRNA.

Parallel to that, another batch of cells was transduced with an empty viral vector to create a proper control cell by making sure that the lentiviral transduction process itself did not influence the production of IL-6 in MDA-MB-231 cells. Those cells were called “non-target” or NT-cells. The success of the transfection was assessed by measuring the IL-6 mRNA levels via real-time PCR and the IL-6 protein levels were measured via ELISA. We found that targeting the IL-6 mRNA at the 636 position yielded the best knockdown results and used the cells targeted at this position for all further experiments. This is why the knockdown cells will be referred to as MDA-IL-636 cells.

3.1.2 Measurement of mRNA levels using real time PCR after knock-down

MDA-MB-231 cells which produce IL-6 constitutively were transduced with a lentivirus containing a plasmid coding for an shRNA that targets the IL-6 mRNA at the nuclear acid bp of the 636 to the 656 position. To assess the IL-6 mRNA level in the knockdown cells we first extracted and purified the IL-6 mRNA and then performed an RT-PCR using the Bio-Rad IQ software to quantify the mRNA knockdown in the parental (MDA-MB-231), non-target (MDA-NT) control cells and the knockdown cells (MDA-IL-6).

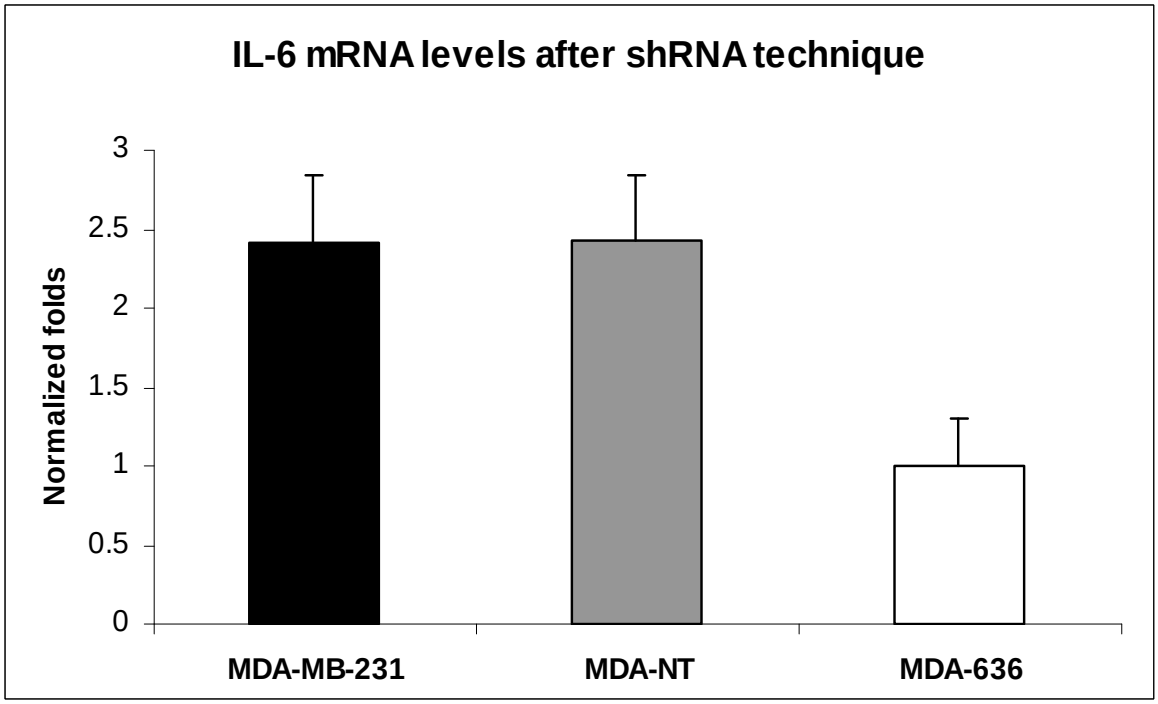


Fig. 4: **mRNA levels**
To prove the success of the knock-down, mRNA-levels were measured in parental (MDA-MB-231), non-target (MDA-NT) and knockdown cells (MDA-IL-6). The mRNA in the knockdown cells was reduced by more than 50% as compared to parental and non-target cells.

According to real time PCR results shown in Fig. 4 L-6 mRNA levels in MDA-636 cells were reduced by more than 50% as compared to the parental MDA-MB-231 cells, whereas the IL-6 level in the MDA-NT cells stayed at about the same level. The knockdown was stable over time.

3.1.3 ELISA

Knock down cells produce lower IL-6 protein levels compared to parental and non-target cells. To further prove that the IL-6 knockdown was successful we decided to measure IL-6 protein levels produced by the cells in the collected conditioned medium using the ELISA technique. We decided to perform the experiment in full serum medium (FSM). As shown on the left side of Fig. 5A the IL-6 protein production in the MDA-636 cells was lowest in the MDA-636 cells.

To exclude the influence of any factors contained in the serum on IL-6 production we decided to measure IL-6 levels after conditioning all cell entities in serum free medium (supplemented with 0.1%BSA).We found that treatment of MDA-MB-231 and MDA-NT cells with serum free 0.1%BSA medium (i.e. cell starvation) leads to a dramatic increase in IL-6

mRNA. Fig. 5B shows the IL-6 protein levels measured in all cell entities after treatment with serum free 0.1% BSA medium. We found that despite the treatment, IL-6 production in the MDA-636 cells was significantly lower in the knock down cells compared to MDA-MB-231 ($p=0.0357$) and MDA-NT ($p=0.0079$) cells and we regard this result as further proof for the stability of our knockdown.

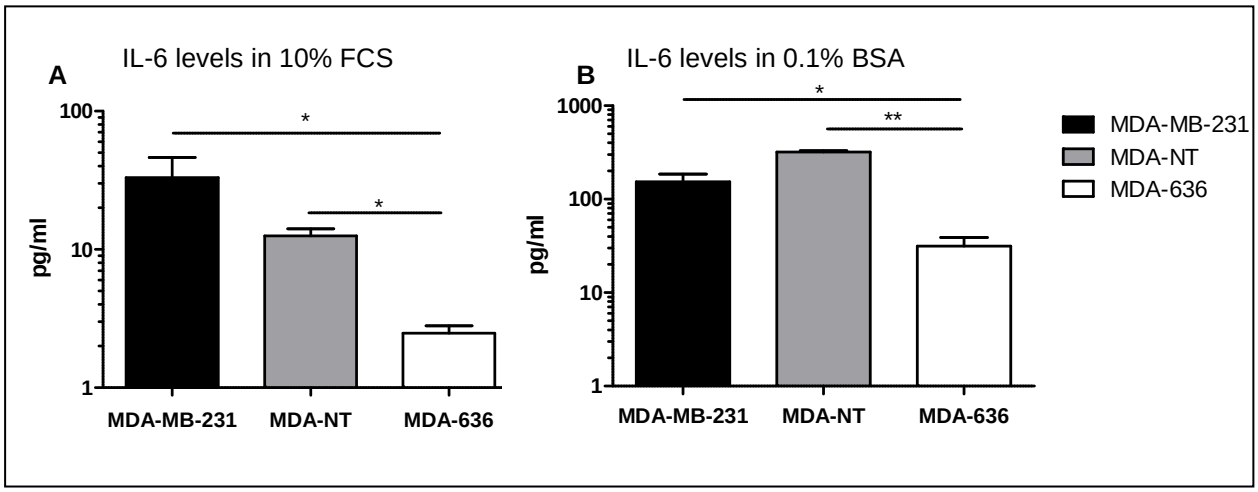


Fig. 5: **ELISA measurements of IL-6 levels in 10% FCS (foetal calf serum) medium and 0.1% BSA (bovine albumin) serum-free medium**

A: IL-6 protein levels of MDA-MB-231, MDA-NT and MDA-636 cells measured in the conditioned full serum medium after 24h incubation. B: shows IL-6 levels from all 3 tested cell entities conditioned in serum free medium. The IL-6 levels in the cells treated with serum free medium are about 10-fold higher than in the cells treated with full serum medium. Despite this treatment with 0.1% BSA serum IL-6 levels in MDA-636 cells stay comparatively low, whereas the levels in the MDA-MB-231 cells and MDA-NT cells go up dramatically which we regard as a proof for the stability of our knockdown.

3.2 *In vitro* cell characterization

3.2.1 Cell growth

IL-6 is not a growth factor for MDA-MB-231 cells *in vitro*. This experiment was designed to evaluate the growth of IL-6 deficient MDA-MB-231 cells compared to the growth of IL-6 sufficient parental cells and non-target cells *in vitro* after proving that the knock down was successful by quantifying the IL-6 mRNA as well as the protein levels. For this purpose we seeded a growth curve of all cell variants in quadruplicates at a concentration of 104 cells per well. A cell count was performed on every day of the experiment and Fig. 6 shows that the cell numbers of all 3 cell entities stayed approximately the same on each day the cell count was performed, with no or little variations. Statistical analysis was performed by 2-way ANOVA and no statistically significant difference in the growth of the 3 cell entities could be detected. As expected the statistical analysis showed a significant growth of cells of each entity over time. To ensure the viability the experiment was repeated three times.

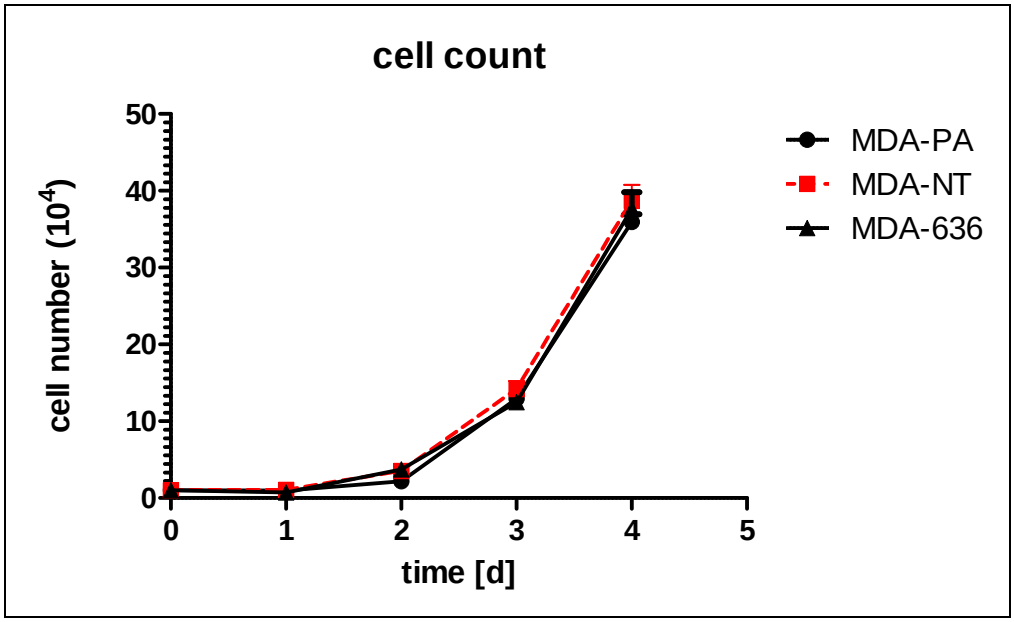


Fig. 6: **Growth curve**
The 3 cells variants, parental (MDA-PA), non-target (MDA-NT) and knockdown (MDA-636) cells were seeded at the same concentration of 104 cells per well in 16-well pates. A cell count was performed daily. Surprisingly, the knockdown of the IL-6 production in MDA-MB-231 cells seems to have no effect on the growth of these cells in vitro, as the cell numbers were almost the same in all 3 cell variants on all 4 days of measurement.

3.2.2 Chemoinvasion assay

To further characterize the knockdown cells we decided to assess their chemoinvasive properties. For this purpose we used a commercially available chemoinvasion assay (for details see methods 2.3.2). The knockdown cells were used as the test cell line and the NT cell line as a control. The cells were seeded into an insert whose porous bottom membrane was occluded by Matrigel. The insert was put into a well with chemoattractive 5% serum medium. Invasive cells have the property to overcome the Matrigel membrane by use of enzymes such as Matrix metalloproteinases. As shown in Fig. 7 the invasiveness of the knockdown cells was reduced to 8.7% as compared to that of the non-target cells which was 19.5%. Statistical analysis using the t-test showed that the reduction of invasive properties in the knockdown cells was significant (p=0.0004)

Fig. 7: **Chemoinvasion assay**
Assesses the of chemoinvasive properties of non-target (MDA-NT) and knock down-cells (MDA-636). Non target-cells had a calculated invasiveness of 19.5 % whereas the knockdown cells had an invasiveness of 8.7%. The reduction of invasive properties for knock down-cells was statistically significant compared to non target-cells (p=0.0004).

3.3 *In vivo* experiments

3.3.1 Assessment of non-target- and knockdown cell growth *in vivo*

The next part of the project consisted of *in vivo* work. As shown in Fig. 8 Balb/c nu/nu nude mice were assigned to 4 groups containing 12 animals each. All groups received a low calcium diet 3 days prior to tumour injection to accelerate bone turnover and fuel the vicious cycle (20). All intratibial injections contained a cell concentration of 10^6 cells per mL phosphate buffered saline and all animals were anaesthetised prior to injections. For intratibial injection the MDA-NT cell was chosen as the control cell to the MDA-636 cell.

Two animal groups were injected intratibially with MDA-NT cells. One of the two groups received a treatment with OPG to block bone turnover and interrupt the vicious cycle, the other received a vehicle only. The remaining two groups received an intratibial injection of MDA-636 cells and again one of the two animal groups received an OPG treatment, whereas the other received a vehicle.

The development of lytic lesions was monitored by digital radiography (MX-20 desktop X-Ray radiograph, Faxitron). The experiment was conducted for 21 days with X-Ray images taken on days 10, 17 and 21. After 10 days, the lytic lesions were clearly visible on the X-Ray images and after 21 days they were quite large. On the 21st day, when the lesions reached a size of approximately 1.5 mm^2 the animals were sacrificed and the tissue was harvested.

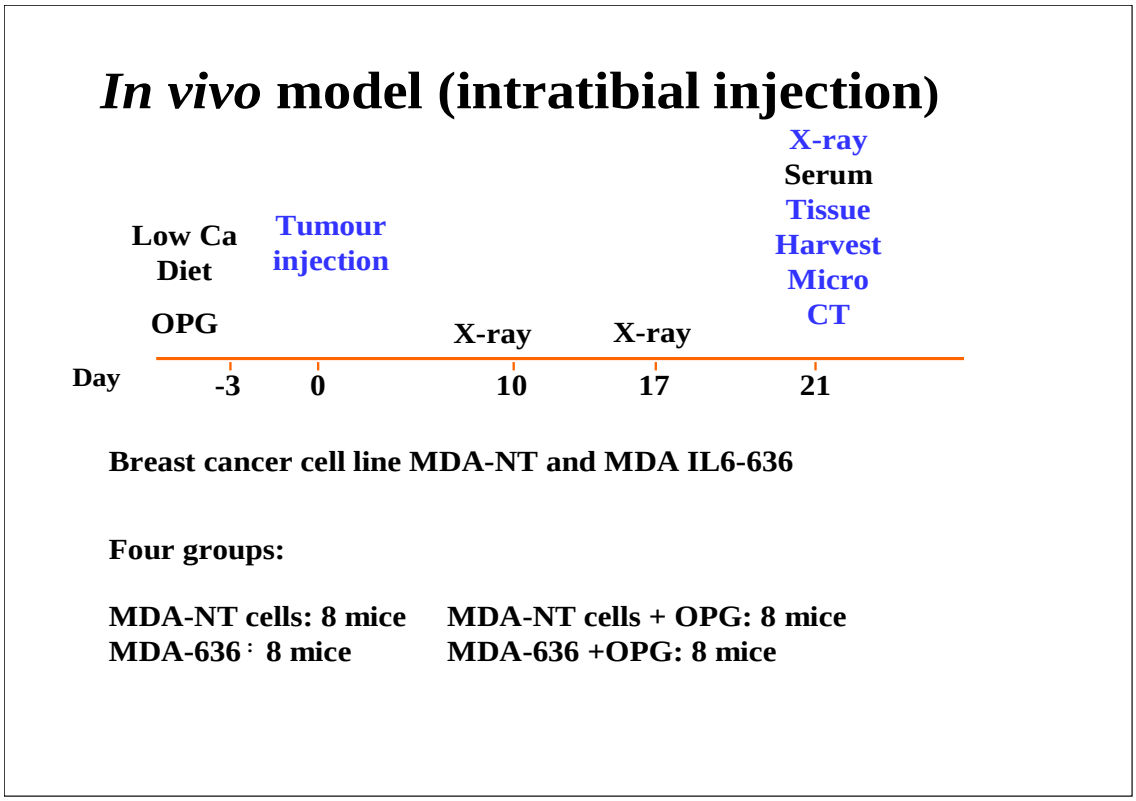


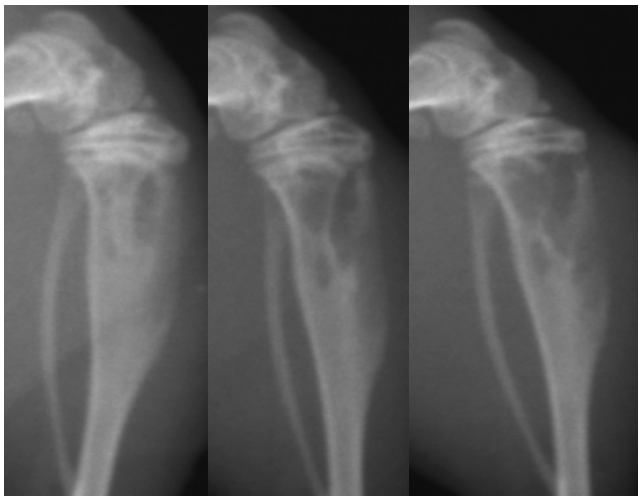
Fig. 8: **In vivo model for intratibial injection**
Shows the experimental setup for the intratibial injection of MDA-NT and MDA-636 cells into 4 groups of nude mice. All groups received a low calcium diet, two groups received the MDA-NT cells, one with a concurrent OPG treatment, two groups received the MDA-636 cells, one of which also received a concurrent OPG treatment.

3.3.2 X-Ray analysis

MDA-636 cells producing low levels of IL-6 show a reduced growth *in vivo* compared to non-target cells. 10 days after tumour cell implantation, lytic bone lesions were detectable by X-Ray in 100% of the non-OPG treated mice. To monitor the tumour growth in the different animal groups X-Ray measurements were performed on days 10, 17 and 21. The MDA-636 and MDA-NT cell injected animal groups that received an OPG treatment did not show any lesions that could be made visible by X-Ray.

A

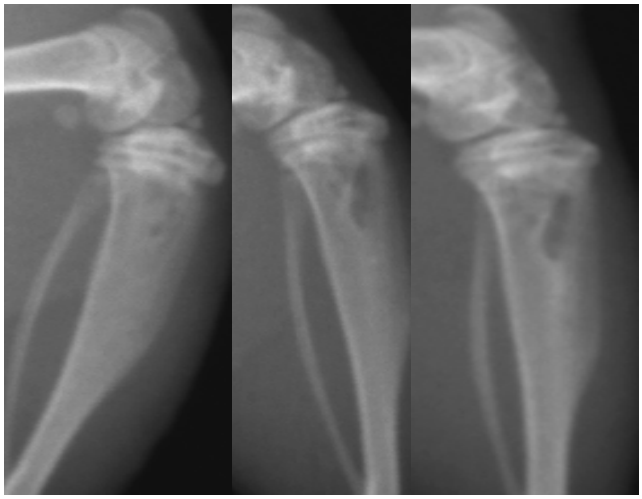
MDA-NT



Day 10 Day 17 Day 21

B

MDA-636



Day 10 Day 17 Day 21

C

X-ray analysis

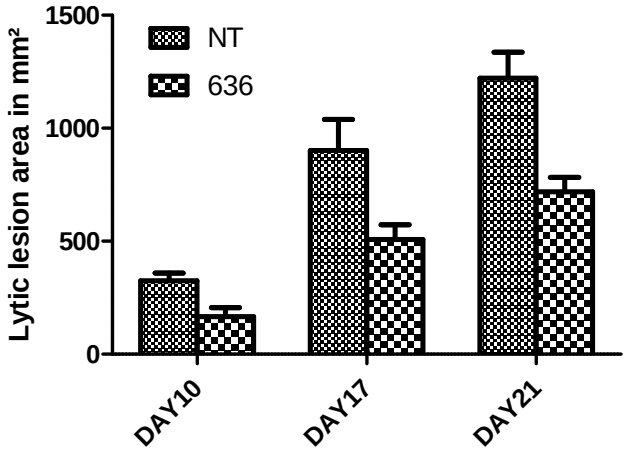


Fig. 9: **X-ray analysis knockdown experiment**
A shows X-Ray pictures of the animal group injected with MDA-NT cells taken on days 10, 17 and 21. The development of the lesions progressed much quicker than in the MDA-636 cell injected group (images taken on days 10, 17 and 21 depicted in B). C shows Tumour growth analyzed through the programme Image J. The lytic lesions developed by the MDA-NT cell injected mice were almost twice as big as those developed by the MDA-636 group on every day that the measurement was performed. As the OPG treated groups did not exhibit any lytic lesions visible on the X-Ray images they were equated to zero and are thus not visible in the graph.

The lesions on the X-Ray pictures of both groups were measured by using Image J for all animals in one group. The mean was calculated for the lesion size of all animals in one group and then graphically depicted (Fig. 9). The pictures of the 2 OPG treated groups were excluded from this analysis as no lesions were visible in them. As can be seen in Fig. 9C the lesions developed by the MDA-636-cell injected mice were almost 50% smaller than in MDA-NT-cell injected mice on every of the 3 days on which the measurement was performed.

3.3.3 Micro-CT pictures

Following sacrifice on day 21, the tibiae were harvested and Micro-CT images were taken. Fig. 10 shows representative images of an animal from every group (Fig. 10A: non-target and knockdown cell injected tibiae, vehicle treated group, Fig. 10B, non-target and knock-down cell injected tibiae, OPG treated group). The Micro CT (µ-CT) measurements confirmed that the animals injected with the knockdown cells developed smaller lytic lesions than the non-target cell injected animals. As can be seen in Fig. 10B lytic lesions were not visualized in the OPG treated animals by µ-CT which confirmed the results seen in the X-ray images.

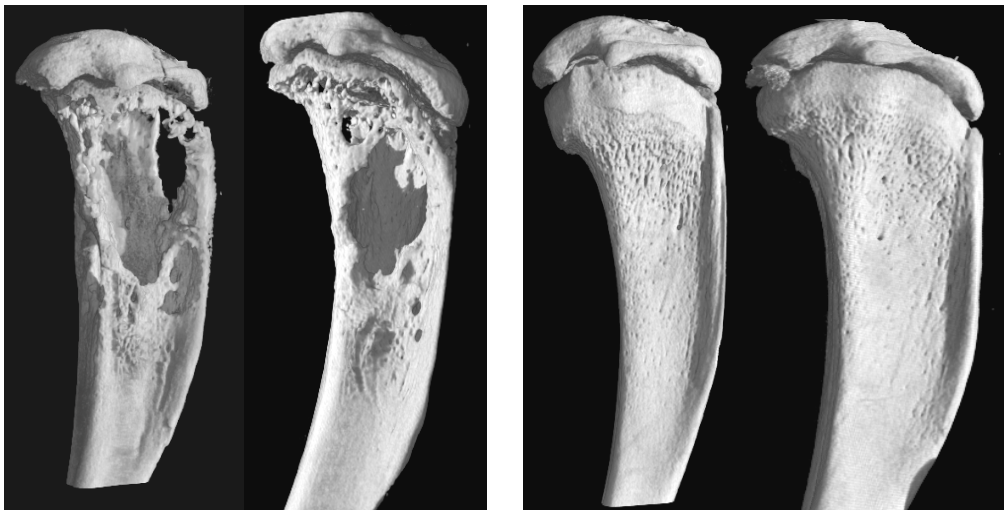


Fig. 10: **µ-CT pictures**
A: Two representative tibiae from either the non-target or knockdown cell injected groups were chosen for this figure. The left side shows an image of an MDA- NT cell injected tibia as opposed to a MDA-636 injected tibia visible on the right side. B shows tibiae of the OPG treated animals from the NT and 636 injected groups. As previously determined with the X-Ray images, no lytic lesions were evident by µ-CT.

3.3.4 Histological analysis

Histological analysis of the harvested tibiae of all four animal groups. The tibiae were decalcified, embedded into paraffin cut and stained with Eosin-Haematoxylin staining and the lytic lesions were measured through bone histomorphometry. The lytic lesion sizes for all animals assigned to each group were added up, averaged and the average was then graphically depicted (Fig. 12). On X-ray and µ-CT images of the OPG treated animals injected with non-target or knockdown cells, no lytic lesions could be detected. Histologically, however, tumour growth could be seen in the OPG-treated groups as shown in Fig. 11 (B and D).

On histological analysis the tibiae of the OPG treated animals also contained tumour tissue but to a much lesser extent and the cortical and trabecular bone was clearly retained. This explains why the tumour lesions could be detected neither by X-Ray nor µ-CT. Histologically there was no difference in the tumour area between the OPG treated MDA-636-cell MDA-NT injected animals.

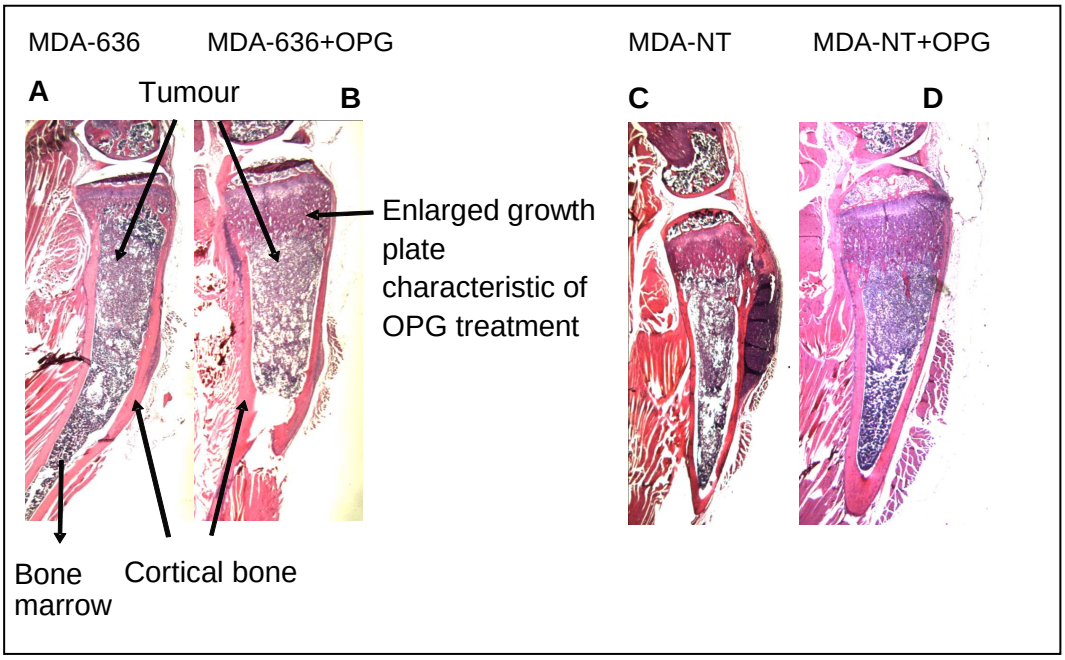


Fig. 11: **Effect of IL-6 knockdown in MDA-MB-231 cells after intratibial injection into nude mice.** Histological view of MDA-MB-231 knockdown cells (A) and non-target cell (C) injected tibiae with (B+D) and without (A+C) OPG treatment (magnification x10). OPG treated tibiae are recognizable by their characteristically enlarged growth plate. In MDA-636-injected non-OPG treated tibiae (A) cortical bone is still mostly intact, the bone marrow cavity still contains bone marrow. In NT-injected non-OPG treated bone (C) almost all trabecular bone is destroyed and cortical bone markedly decreased. The bone marrow cavity is completely replaced by NT-cells. In OPG treated tibiae regardless of the cell entity injected (B+D) cortical and trabecular bones are still intact, which is why radiographically no lytic lesions could be detected even though the tibiae contain tumour tissue.

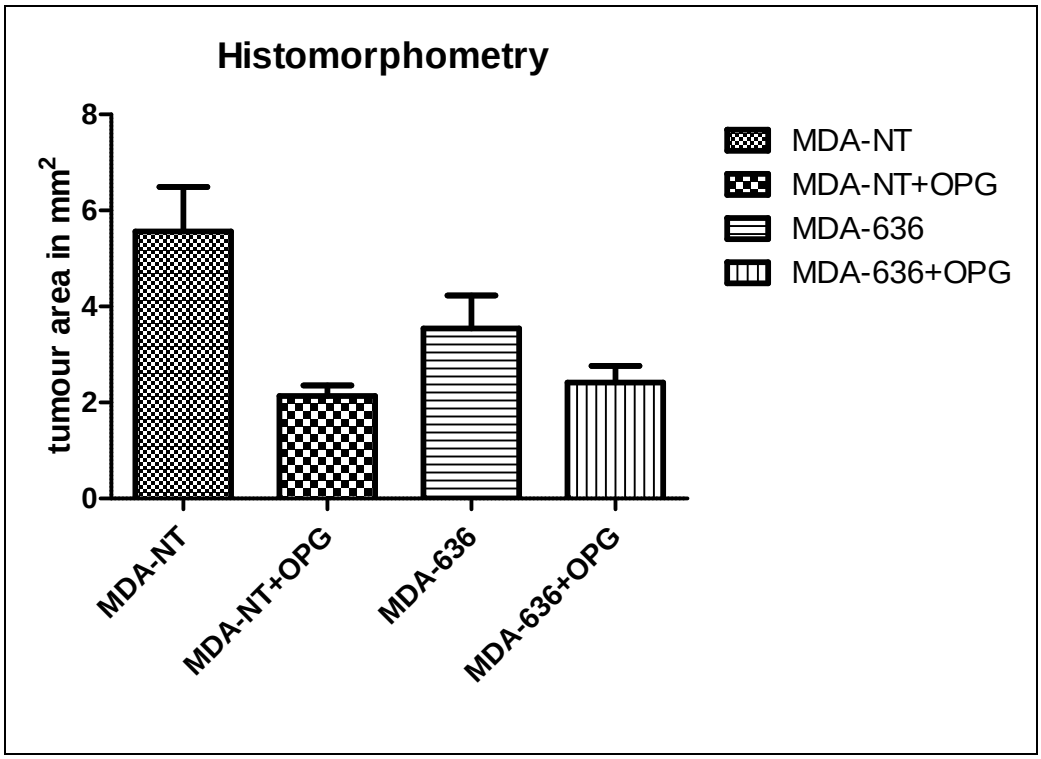


Fig. 12: **Histomorphometric analysis**
Histologic analysis of tumour burden in mice injected with MDA-NT or MDA-636 cells and with or without OPG treatment confirmed the results of the X-ray measurements. The tumour burden in the knockdown cells was significantly reduced by approximately 50% in the knockdown cells as compared to the non-target cells. OPG treatments lead to a reduction of tumour growth regardless of the cell entity.

3.4 Mammary fat pad injection

To investigate the effect of the IL-6 knock down on MDA-MB-231 cell growth away from the bone microenvironment, we decided to inject balb/c nu/nu mice with the non-target and knockdown cells subcutaneously into the mammary fat pad. The cells were suspended in a mixture of PBS and Matrigel and injected into the animals. Tumour growth was assessed daily by measuring the length, width and height of the tumours and the tumour volume was calculated by using the formula for ellipsoid bodies $4/3\pi$ x width x length x height. We started measurement on the 2nd day to give the Matrigel time to dissolve to avoid artefactual measurements caused by this substance.

3.4.1 Comparison of knockdown and non-target cell growth in mammary fat pad

In the mammary fat pad there was no difference in tumour growth between knockdown and non-target cells. Tumours were palpable from day one after implantation into the mammary fat pad. Measurements were undertaken on every 2nd day. During the following 16 days of the experiment, tumour growth stayed the same in MDA-NT and MDA-636 cell injected animals. As can be seen in Fig.

13A tumours of both cell entities exhibited the same growth rate on each day the measurement was performed. Knocking down the IL-6 production in MDA-MB-231 cells thus had no effect on the tumour growth away from the bone microenvironment and the vicious cycle. On day 16 the animals were sacrificed and the tissue was harvested. The tumours were weighed, the results were added up and averaged (Fig. 13B).

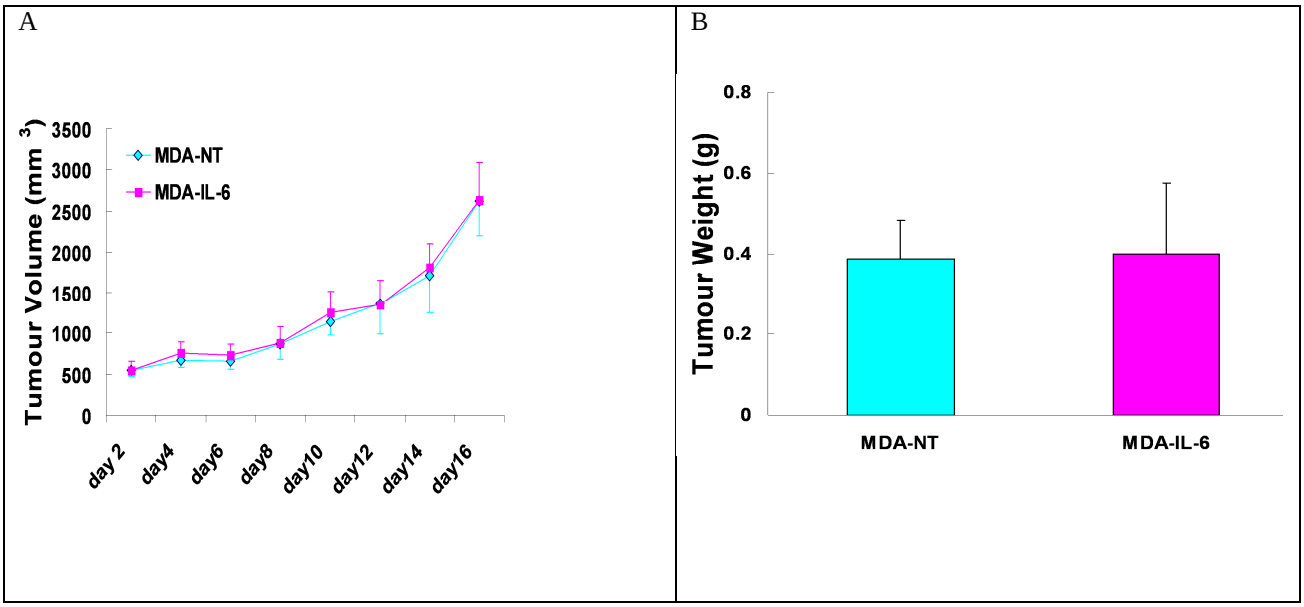


Fig. 13: **Mammary fat pad injection**
Effect of IL-6 knockdown on subcutaneous tumour growth. A: Tumours were palpable from day one after implantation into the mammary fat pad. Measurements were undertaken on every 2nd day. During the following 16 days of the experiment tumour growth stayed the same in MDA-NT and MDA-636 cell injected animals. B: On day 16 the animals were sacrificed and the tumour tissue was harvested. The bar graph displays the tumour weight of the non-target and knockdown groups (mean±SD).

3.5 *In vivo* results for animal experiment with Tocilizumab

After we found that knocking down the IL-6 production in MDA-MB-231 cells leads to the development of smaller tumours *in vivo*, we decided to conduct a second *in vivo* experiment using the humanized IL-6R antibody Tocilizumab. As can be seen in Fig. 14 MDA-MB-231 cells produce human IL-6 which interacts with the human IL-6R on the tumour cell surface but also with the murine IL-6 R on the surface of the osteoblast and the soluble murine IL-6R in the system of the mouse whereas murine IL-6 from the murine bone microenvironment cannot interact with the human IL-6R on the tumour cell surface (57), (28). By injecting a human tumour cell line into the bone microenvironment we thus created a setup where the only IL-6 that interacted with the human IL-6R on the surface of the MDA-MB-231 cell was the IL-6 produced by the cell itself. According to Grivennikov *et al* IL-6 is an autocrine and paracrine growth factor for breast cancer cells (58). We assumed that blocking the autocrine and paracrine stimulation of the MDA-MB-231 cells by Tocilizumab would lead to decreased breast cancer cell growth in bone.

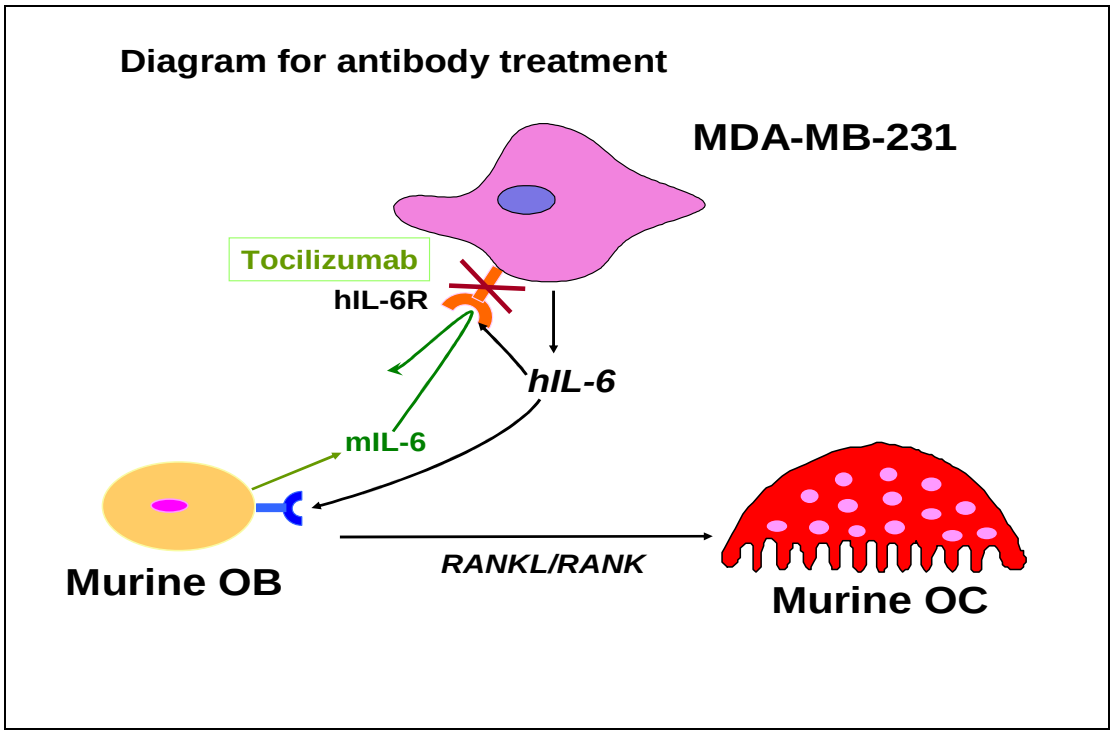


Fig. 14: **Diagram for antibody treatment**
Shows how the human IL-6 produced by the MDA-MB-231 cell interacts with the human IL-6R on the tumour cell surface and the murine IL-6R on the murine osteoblast. The murine bone environment derived IL-6, however, cannot interact with the human IL-6R on the tumour cell surface. Administration of Tocilizumab thus only inhibits autocrine and paracrine IL-6 interaction with IL-6R on the tumour cell surface and creates a possibility to establish the importance of autocrine and paracrine IL-6 signalling in breast cancer cell growth in bone.

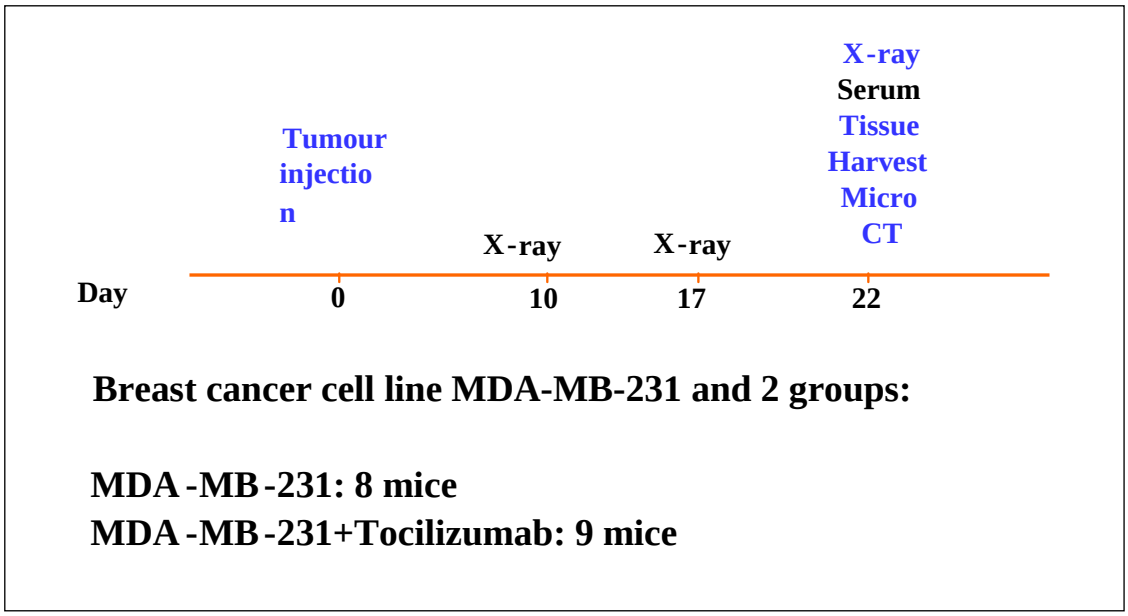


Fig. 15: **Time-line Tocilizumab experiment**
Inhibition of IL-6R signalling in tumour cells by Tocilizumab. The human IL-6R antibody Tocilizumab was administered to mice injected intratibially with MDA-MB-231 cells to prevent the autocrine stimulation of tumour growth and IL-6 production in these cells.

For the Tocilizumab experiment, balb/c nu/nu mice were assigned to two groups containing eight and nine animals, both of which received intratibial injections of MDA-MB-231 cells. The nine-animal group received a Tocilizumab treatment, whereas the eight-animal group did not receive any treatment at all. The experiment was conducted for 22 days with X-Ray images being taken on day 10, 17 and 22 to monitor the tumour growth in the animals. On the 22nd day the animals were sacrificed and the tissue was harvested (Fig. 15).

3.5.1 Tocilizumab reduces cancer growth in bone

Treatment of MDA-MB-231 cells with the humanized IL-6R antibody Tocilizumab reduced breast cancer cell growth in bone. On each day when the X-Ray measurements were performed, the lytic lesions in the in the Tocilizumab treated group were smaller than in the control group. Fig. 16 shows X-Ray and μ-CT picture for one representative animal from each of the two groups.

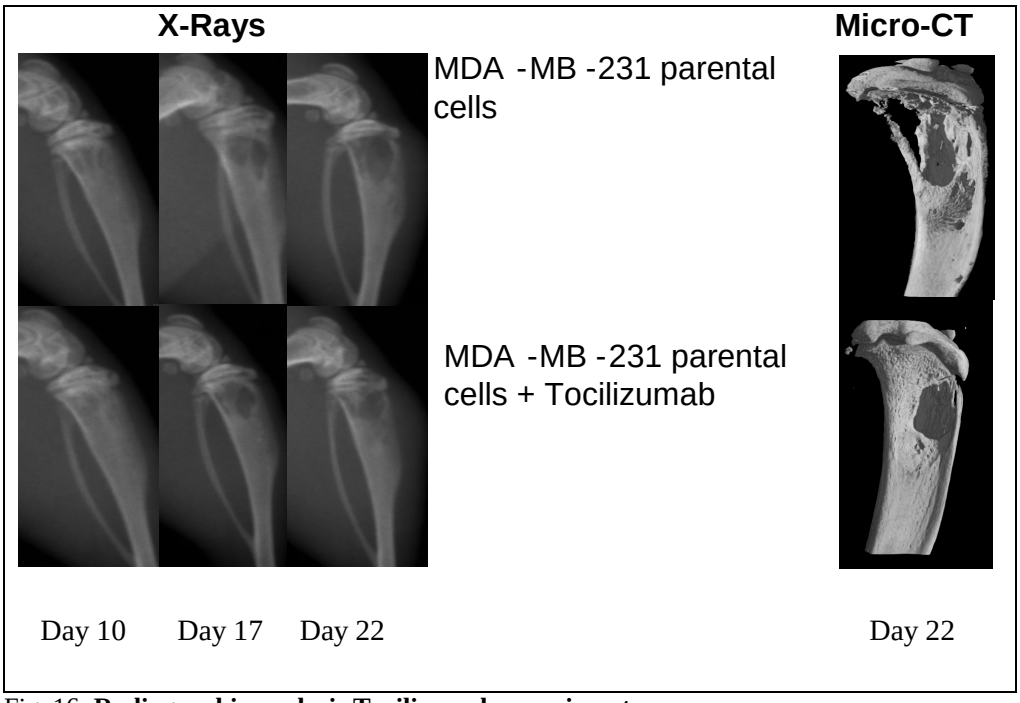


Fig. 16: **Radiographic analysis Tocilizumab experiment**
On the left hand side X-Ray images of 2 representative animals from the control group (upper half) and Tocilizumab treated group (lower half) are shown on day 10, 17 and 22. After the animals were sacrificed on day 22 μ-CT of the tibiae were performed, the result of which is displayed on the right side of the figure.

The X-Ray images taken on day 10, 17 and 22 of all animals in one group were analysed with the programme Image J. The lytic lesion area was measured for all animals in the group, added up and averaged and the result was depicted graphically as can be seen in Fig. 17.

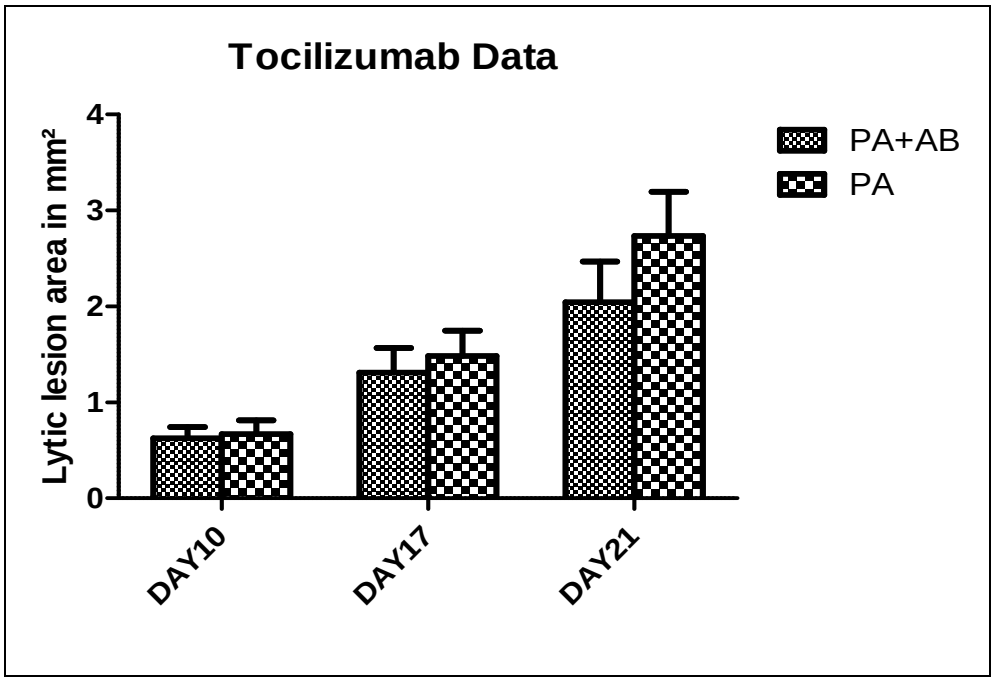


Fig. 17: **X-Ray analysis Tocilizumab experiment**
X-ray images were taken on day 10, 17 and 21 and the lytic lesion area anayzed by means of the programme Image J. The lytic lesion area in the Tocilizumab treated animals was numerically smaller on every day of measurement than in the control group. The result, however, was not statistically significant (p=0.08).

4 DISCUSSION

Breast cancer associated bone metastasis remains a common cause of morbidity and mortality for affected patients. After metastatic breast cancer is diagnosed, the life expectancy of the patient is reduced to approximately 20 months (59). Gregory Mundy's model of the vicious cycle is so far the best way to explain why bone provides a fertile soil for metastatic breast cancer (11). All factors that contribute to the thriving of tumour cells in the bone microenvironment, however, are not yet completely known. It is important to fully elucidate and identify the factors responsible for the development of bone metastasis to create better treatment opportunities, improve the patient's quality of life and maybe one day create a remedy for this hitherto incurable disease.

It is known from clinical experience that breast cancer patients presenting with high serum IL-6 face unfavourable clinical outcomes and high mortality rates (28), (36). IL-6 producing breast cancer cells, like the ER (-) MDA-MB-231 cell line, are highly malignant, metastatic and prefer bone as their metastatic site. We hypothesized that IL-6 might be one of the factors responsible for the thriving of breast cancer cells and the accelerated tumour growth in the bone micro-environment. It was the objective of this work to investigate the role of IL-6 in breast cancer cell growth in the bone microenvironment.

It was shown in this thesis that the production of the IL-6 mRNA in MDA-MB-231 cells could be knocked down by about 50% using the shRNA transfection technique and that this led to a reduction in IL-6 protein production by about 70–80% in the knockdown cells. The discrepancy in IL-6 mRNA levels and actual protein levels might be explained as follows: by using the shRNA technique we were able to reduce IL-6 mRNA levels by 50%. This led to a reduction in IL-6 protein production by the knockdown cells. The lower IL-6 protein levels produced by these cells might have, in turn, led to a lesser autocrine and paracrine stimulation of the cells to produce IL-6. This would correspond to the observation made by Gao *et al*. for lung cancer cells that IL-6 stimulates its own production in an autocrine and paracrine manner (60).

When assessing the growth rate of the knockdown cells as compared to the parental and non-target cells, we realized that the knockdown had no effect on the growth behaviour of these cells *in vitro*. This finding might be due to the fact that the knockdown of the IL-6 production achieved through shRNA technique was incomplete and that the remaining IL-6 was still able to stimulate the breast cancer cell growth in the knockdown cells.

Sasser *et al*. showed that adding exogenous IL-6 to MCF-7 cells that normally do not produce IL-6 leads to increased growth and malignancy in these cells but were unable to show

the same effect for the IL-6 producing cell line MDA-MB-231. Sasser *et al*. explained their results through STAT3-phosphorylation which occurred in MCF-7 cells after adding IL-6 but was constant in the MDA cells (28). It is thus likely that IL-6 produced by the MDA cells leads to constant STAT3 activation in an autocrine and paracrine manner, to which adding exogenous IL-6 does not contribute. STAT3 activation in turn is believed responsible for more increased tumour growth and malignancy. It is hence possible that in our experiment the remaining IL-6 whose production we were unable to knock down, led to sufficient STAT3 phosphorylation and thus unchanged growth in our knockdown cells. It would be interesting to check if in the knockdown cell STAT3 is still activated. If that is the case it would explain why we were unable to see any changes in growth in the knockdown cells.

Moreover, for the cell growth experiment the cells had to be held in full serum medium as serum free medium led to cell death on the 2nd or 3rd day. It would thus have been impossible to conduct the counting for four days and provide significant results. By using full serum medium we were, however, unable to exclude the possible effects of different growth factors on STAT3 this medium contains in the knockdown cell.

Another possibility is that endogenous IL-6 is not a growth factor for MDA-MB-231 cells and the reduction of tumour growth in the knockdown cells is indirect through reduction of bone resorption, reduction of growth factor release from the bone matrix and thus reduced stimulation of tumour growth (vicious cycle). This would correspond with the observation that IL-6 is not an exogenous growth factor in ER(-), IL-6 producing breast cancer cells, made by Sasser *et al*. and our own observation that knockdown of IL-6 did not lead to reduction of tumour growth in the mammary fat pad, away from the bone microenvironment.

Selander *et al*. conducted a study in which they blocked IL-6R signalling through a gp130 inhibiting molecule. This led to the inhibition of the effects of almost all members of the IL-6 family on the MDA cells, including IL-8, which signal through the IL6-receptor and thus excluded the influence of all those family members on the cancer cells. They could show that in the mammary fad pad model MDA-MB-231 cells with abrogated IL-6R signalling were much less invasive. They assume that since inhibiting IL6-R signalling leads to the up-regulation of metallopeptidase inhibitor 3 (TIMP-3), an inhibitor of VEGF and matrix metallopeptidase (MMP-9), the cells become less invasive (47). In our case knocking down the IL-6 production in MDA-MB-231 cells could have also led to decreased gp130 activation and thus up-regulation of TIMP-3. If this were the case, TIMP-3 would lead to a decreased MMP-9 activity in the knockdown cells and provide an explanatory mechanism for the decreased invasiveness of the knockdown cells that we saw *in vitro* using a commercially available chemoinvasion assay. We

were able to show that the knockdown cells were only half as invasive as the non-target cell used as a control.

Several RT-PCR measurements were performed in the knockdown cells trying to elucidate whether the knockdown had either up-regulated the TIMP-3 mRNA levels or down-regulated the MMP-9 or VEGF mRNA levels but were unable to show any correlation (data not shown). This could be due to the fact that by implementing the knockdown technique we were only able to reduce IL-6 production and not completely abrogate it. The remaining IL-6 produced by the knockdown cells can still activate the IL-6R. Moreover the other members of the IL-6 family weren't affected in our experiments, which is another difference to the experiments by Selander *et al.* The reduction of IL-6 signalling might not be enough to influence such downstream factors as TIMP3, MMP9 and VEGF, whereas the complete abrogation of its production might.

Matsumoto *et al*. used a NFκB inhibitor called dehydroxymethylepoxyquinomicin (DHMEQ) that abrogates the constitutive NFκB activation and effect of TNFα on MDA-MB-231 cells. This led to a decrease in production of IL-6 and IL-8 by more than 50% and a significant reduction in tumour volume *in vivo* compared to DHMEQ free controls. NFκB has also been shown to up-regulate the expression of several proangiogenic genes, directly or indirectly, including urokinase-type plasminogen activator, MMP9, and vascular endothelial growth factor (37). Since NFκB is also a possible factor downstream of the IL-6R (61), it would be interesting to test whether in our knockdown cells the activity of NFκB is reduced compared to the parental cells and whether that is in any way related to the reduced intratibial tumour growth we saw in our experiments.

So how can the effect of smaller tumours that we saw in our *in vivo* intratibial experiments with the knockdown cells or Tocilizumab be explained if the reducing IL-6 production did not lead to a significant reduction of growth or malignancy in the MDA cells?

A possible explanation that comes to mind is that the key factor is the vicious cycle that constitutes the difference between the settings of the mammary fat pad and the tibia and explains why bone is the preferred metastatic site of breast cancer metastasis. To answer this question in a satisfying way, however, a closer look needs to be taken at what is already known about IL-6 and its influence on different participants in the vicious cycle.

As will be explained extensively later on the autocrine and paracrine IL-6 from the tumour cells is the only one able to interact with the IL-6R s on the tumour cell surface, whereas murine IL-6 is not. The reduction of the IL-6 production must have led to decreased stimulation of the IL-6R and thus a decrease in STAT3-activation. One possibility why this did not translate

into decreased tumour growth in the mammary fad pad, as we expected according to the findings mentioned by Grivennikov *et al*. (58) is the following: As we showed in our ELISA experiment, submission of the knockdown cells to starvation, in other words submitting the cells to stress, led to increased IL-6 production in all cell entities tested. Even though in the MDA-636 cells IL-6 levels were about 9-fold lower than in the two control cells, starvation/stress was still able to induce IL-6 production. This is most likely due to the fact that we were unable to completely knockdown IL-6 mRNA even though it was reduced by 50% as compared to our control cells and that the knockdown cells still produced measurable IL-6 protein levels.

Most likely injection of the knockdown cells into the mammary fat pad of nude mice would have meant stressing the cells which could have led to increased IL-6 levels in our knockdown cells and increased STAT3 activation. In this setup, away from the bone microenvironment and the vicious cycle, the only effect that would have led to reduced tumour growth would have been the reduction of such downstream factors of the IL-6R as TIMP3, MMP-9 and VEGF. If there is such an effect of the IL-6 knockdown on these factors, it might have been abrogated because as seen in our ELISA experiment the induction of IL-6 through stress is still possible in our knockdown cells even if to a lesser extent.

Even though we were unable to do so, it would be interesting to create a MDA-636 cell with a complete IL-6 knockdown and see whether the growth of this cell will be reduced in the mammary fat pad.

4.1 IL-6 and the vicious cycle

Breast cancer cells are known to produce different factors such as PTHrP, TNFα, IL-6 and 11 (62) which induce osteoclastogenesis. The overproduction of PTHrP by breast cancer cells in the bone microenvironment leads to a hyperparathyroidism-like state consisting of increased RANKL production by the osteoblast, increased osteoclast and lacunar resorptive activity (4). Moreover, PTHrP increases the secretion of RANKL in osteoblasts which leads to increased osteoclast differentiation through interaction with RANK on the osteoclast surface (11). Thomas *et al*. reported that the induction of RANKL in the osteoblast was concomitant to the reduction of OPG production.

After activating osteoclasts through RANK/RANKL interaction, active osteoclasts degrade the bone matrix thereby releasing growth factors like IGF-1, TGF-β, FGF and Ca^{2+} which contribute to cancer proliferation and increase the PTHrP secretion from the cancer cell. This was backed up by the finding that cancer cells stably transfected with mutant TGFβ

receptors unresponsive to TGFβ do not produce PTHrP, and cause significantly less osteolytic lesions after injection into nude mice (18). The process of increased tumour proliferation through factors released from degraded bone was termed the “vicious cycle” (11).

Within the vicious cycle IL-6 and PTHrP and IL-6 act synergistically: Firstly, IL-6 contributes to the vicious cycle by stimulating RANKL production in osteoblasts. This effect is mediated via sIL-6R as the osteoblast expresses only a small amount of membrane bound IL-6R (17) (63). Secondly, IL-6 lowers the levels of OPG produced by the osteoblast, which in turn increases RANK/RANKL interaction and leads to increased osteoclast activation (63). Thirdly, it has been reported that IL-6 promotes bone resorption by increasing PTHrP production in osteoblasts (51).

To sum up, IL-6 supports the vicious cycle in different ways like: contributing to bone resorption through RANK/RANKL mediated osteoclast activation. This leads to increased degradation of bone and release of growth factors from the bone matrix which in turn increases the PTHrP secretion from tumour cells. Moreover, IL-6 is able to directly increase the PTHrP secretion from tumour cells and osteoblasts (51).

4.2 IL-6 and the osteoclast

IL-6 increases bone resorption in the following pathological conditions: Paget’s disease (64, 65), Multiple Myeloma (66), Gorham-Stout disease (67), estrogen-deficiency induced bone loss (68) and of course breast cancer.

It is, however, debatable whether IL-6 has a direct effect on osteoclast formation and activation or whether the effect is indirectly mediated by increasing the RANKL production in osteoblasts and PTHrP production in breast cancer cells.

Several *in vitro* studies investigated the role of different breast cancer cell-produced cytokines including IL-6 on osteoclast formation. Whereas Manelagas *et al*. stated that IL-6 requires cells of stromal/osteoblastic origin to induce osteoclast formation, Kudo *et al*. designed an study where IL-6 together with soluble IL-6R and MCSF were added to CD-14^{+} human peripheral blood mononuclear cells and induced the formation of TRAP, VNR and calcitonin receptor positive cells capable of lacunar bone resorption. This reaction could be inhibited by anti gp130 but not OPG or RANK-Fc implying that there is a RANKL-independent mechanism of osteoclast formation through IL-6 if the sIL6-R is present. According to this study M-CSF and IL-6 are crucial factors in osteoclast formation and this is a parallel mechanism to osteoclast

formation through RANK/RANKL interaction. It seems that this role, however, is of minor importance since blocking the RANKL dependent osteoclast activation by OPG leads to the complete absence of osteoclasts (5) .

Morgan *et al*. designed a coculture model where different breast cancer cell lines including the MDA-MB-231 cells were co-cultured with spleen cells and osteoblasts, which led to osteoclast formation. The production of IL-6, IL-11, TNFα and PTHrP in the tumour cells was stimulated through the addition of TGF-β which is the growth factor released from bone after osteoclast induced bone resorption. Then antibodies to the cytokines produced by the tumour cells were added but the only antibody that inhibited osteoclast formation was that to PTHrP. An antibody to IL-6 failed to inhibit osteoclast formation (69) which contradicts the result obtained by Kudo *et al*. A limitation to the study by Morgan *et al*. is that no soluble IL-6R was added to the coculture system. As osteoclasts do not express a membrane bound IL-6R (70, 71) the IL-6 produced by the breast cancer cells could not interact with the osteoclast in the first place and thus the administration of an IL-6 antibody did not reduce osteoclast formation.

According to these contradictory results it is debatable whether IL-6 contributes to osteoclast formation by a direct mechanism as described by Kudo *et al*. or only by indirect mechanism as described for instance by Liu *et al*. The important thing, however, is that it does contribute to osteoclast activation and that this osteoclast activation leads to increased bone resorption, release of tumour growth promoting growth factors from the bone and thus enforcement of the vicious cycle.

When relating these findings to the experiments that are the object of this work, it becomes likely that the tumour inhibiting effects seen in the intratibial *in vivo* experiments are due to the reduced IL-6 production by the MDA-cells, which led to reduction in RANKL and subsequently to reduced osteoclast activation.

4.3 IL-6 and the bone microenvironment

The tumour microenvironment in the bone contains different cells that contribute to the tumour development such as bone marrow stromal cells, especially fibroblasts, immune cells like monocytes/macrophages, osteoblasts and the extracellular matrix. Fibroblasts in tumours have been termed “carcinoma associated fibroblasts” and contribute to tumour development by producing Extracellular matrix (ECM), which provides a scaffold for cancer progression, and growth promoting factors like VEGF. Moreover, together with osteoblasts they produce RANKL which is a factor critical for osteoclastogenesis (72).

Other cells that are capable of IL-6 production in the bone microenvironment are bone marrow stromal cells, osteoblasts and monocytes/macrophages (46). According to Bellido *et al*. IL-6 reduces apoptosis in osteoblastic cells through the activation of the p21 gene and contributes to the maturation of committed osteoblastic cells via JAK/STAT activation (25, 73, 74).

IL-6 increases the secretion of PTHrP from osteoblasts in a non-cancerous setting. Guillén *et al* showed that IL-6 together with IL-6sR rapidly increased PTHrP mRNA in human osteoblastic osteosarcoma MG-63 cells and human osteoblastic cells from trabecular bone (51). Osteoblasts express the IL-6R s on a low level only. It has been established that IL-6 exerts its effect on the osteoblast mainly through the sIL-6R. Since IL-6 can increase the PTHrP secretion from osteoblasts in a non-cancerous environment, it is possible that the same happens in a cancerous environment, where the IL-6 produced by the cancer cells could lead to PTHrP secretion from the osteoblast. If this were the case, this would be yet another way for breast cancer derived IL-6 to contribute to bone resorption and the formation of osteolytic lesions.

In another non-cancerous setting it was shown that IL-6 markedly potentiates the effects of PTHrP. IL-6 appears to enhance PTHrP-mediated bone resorption by increasing the pool of early osteoclast precursors that in turn can differentiate to mature osteoclasts. *In vitro* studies have suggested that the effect of IL-6 on osteoclastogenesis may be necessary to mediate PTH-rP-stimulated bone resorption (75). If this finding is applicable to the breast cancer bone metastasis setting this provides additional proof of the enhancing role we assume IL-6 to play in the vicious cycle.

In a transgenic mouse model of rheumatoid arthritis that overexpressed IL-6, a decrease in bone formation in the developing skeletal system was found. IL-6 reduced the osteoblast activity and increased the expression of the BMP gene (76). Applied to the situation in cancer metastasis to bone it provides a possible explanation as to why the bone repair is impaired and the replacing bone is of inferior quality.

In the *in vivo* experiments described in this work human breast cancer cells were injected into nude mice. It has been known for some time now that murine IL-6 cannot interact with the human IL-6R, whereas human IL-6 can interact with the murine IL-6R (57), (28). So by using the model of a human cancer in a murine environment a situation was created where the mouse-derived IL-6 had no effect on the cancer cells whereas the human tumour produced IL-6 did have an effect on the murine bone microenvironment. Thus murine IL-6 could not influence the knockdown cells for instance by enhancing IL-6 production.

We thus assume that the reduction of IL-6 in the MDA cells was not altered by the murine bone microenvironment and the production of IL-6 from those cells stayed as low as that which we measured *in vitro*. We also assume that the low IL-6 levels lead to decreased tumour growth in bone through their missing enforcement on the murine vicious cycle since the same effect could not be reproduced in the mammary fat pad away from the bone microenvironment.

The exclusion of murine IL-6 from influence on the tumour cell is thus an artificial environment that cannot be found in breast cancer patients since in the human body the bone microenvironment can very well influence the human tumour. It can, however, be seen as a positive side effect to an experimental limitation because it allows us to have a look at breast cancer and its influence on the environment of the host without the host being able to influence the tumour. The conclusions we derive from the unilateral setup can be seen as direct tumour effects on the bone microenvironment without any reciprocating effects from the environment itself and thus allow a dissection of the involved mechanisms.

In a patient with breast cancer the situation would of course be different since the bone derived IL-6 can very well interact with the tumour cell. But in the humanized IL-6R antibody Tocilizumab we have a potent drug that inhibits any IL-6R/IL-6 interaction and would thus also prevent bone derived IL-6 from interacting with the tumour cell.

4.4 IL-6 and the oestrogen receptor

ERα-positive breast cancer cell lines like the MCF-7 cells are knows to be less malignant than their ERα-negative counterparts. Bandyopadhyay *et al*. stably transfected the MDA-MB-231 cell with the ER and found that those cells showed both significantly reduced homing to bone and osteolytic potential. They hypothesize that this effect could be due to the reduction of RANK on the MDA cell surface (43). Since estradiol antagonizes IL-6 function by repressing both IL-6 and its signalling receptor gp130 (77) it could be also possible that the effects seen in the MDA cells after ER transfection could be due to reduced IL-6 production. The authors did not measure IL-6 levels produced by their cells, so this is merely an assumption that would be interesting to investigate.

4.5 Future work

4.5.1 Interleukin- 6 and immune evasion in bone metastasis

The members of the TNF family relevant in bone physiology, specifically osteoclast formation and cell function, include the aforementioned TNFα, RANKL and OPG (78).

As mentioned earlier, OPG is a soluble decoy receptor for RANKL produced by osteoblasts and is a pivotal factor in regulating RANKL thus preventing uncontrolled bone resorption.

TRAIL is another member of the TNF superfamily which can activate a cascade leading to the recruitment and binding of intracellular components of the death-inducing signaling complex (DISC) and thus cell death. Blocking TRAIL activity *in vivo* led to promotion of tumour development and increased liver metastasis, suggesting a role in immune surveillance for TRAIL (79).

MDA-MB-231 cells have been reported to produce OPG. OPG binding of TRAIL by tumour produced OPG could be a possible mechanism of apoptosis evasion by tumour cells (78). OPG was found to have a similar affinity to both RANKL and TRAIL (78).

It would be interesting to investigate whether there is a correlation between IL-6 and OPG production in our knockdown cells and whether by reducing IL-6 we could also reduce OPG. If that were the case it would be another explanation why we saw reduced tumour growth in the knockdown cell experiment, as reduction of tumour produced OPG would make the cells more susceptible to TRAIL and lead to increased cell apoptosis.

However, as mentioned earlier, the result of the mammary fat pad experiment makes it highly probable that the tumour promoting role of IL-6 in breast cancer bone metastasis is mediated through its involvement in and support of the vicious cycle since without them the IL-6 deficient knockdown cell tumour grew as quickly as their IL-6 sufficient counterparts.

4.5.2 Experiments with the murine IL-6R antibody MR16-1

In the experiment with the humanized antibody Tocilizumab we inhibited autocrine and paracrine stimulation of the MDA-MB-231 cells in the bone micro-environment. Murine IL-6 is not able to interact with the human IL-6R but human IL-6 is able to interact with the murine IL-6R (28).

Since a murine IL-6R antibody called MR16-1 is available for research purposes it would be interesting to conduct an experiment where the effects of human IL-6 on the murine bone microenvironment would be abrogated by the administration of this antibody. According to the results we were already able to obtain in the Tocilizumab experiment described above, this should lead to a significant reduction in tumour growth.

The next step could be an experiment where a combination of both antibodies would lead to complete inhibition of the IL-6 influence in the murine bone microenvironment and should yield an even more reduced tumour growth. Moreover, this setup would make the creation of an MDA-MB-231 cell with a better knockdown result than we could already obtain superfluous.

The conclusions derived from this experiment might even be more reliable when compared to the knockdown experiments, since it is known that it is difficult to control the knockdown technique and limit the knockdown to the target gene only. When implementing the knockdown technique, up to 10% of the mRNAs of other genes might be affected in the transfection process which is an important limitation to that technique (80).

4.5.3 Clinical relevance of the project

According to our findings IL-6 plays an important role in breast cancer cell growth within the bone microenvironment. IL-6 producing MDA-MB-231 breast cancer cells are known to be more malignant, aggressive and metastatic than their IL-6 deficient counterparts, (43) and show reduced growth in bone *in vivo* if the IL-6 production is knocked-down. Bandyopadhyay *et al*. transfected the MDA-MB-231 cell line with an ectopic estrogen receptor. They did not measure IL-6 levels in the transfected cells but since, as mentioned above, IL-6 secretion is regulated by the ER it likely that these cells produced less or no IL-6. These cells showed a reduced homing to bone and decreased osteolytic potential, a fact that might be due to decreased IL-6 production. Future work will show whether IL-6 is also important for the homing of breast cancer cells to bone or for their development of multidrug-resistance.

Since the humanized IL-6R antibody Tocilizumab is now clinically available it would be a possibility to administer it to patients with high IL-6 serum levels and bad clinical prognosis. It could then be assessed whether Tocilizumab leads to any reduction in metastasis formation or growth in the bone microenvironment and thus improvement of quality of life for the patients and disease outcome. In humans, Tocilizumab would inhibit both the autocrine

stimulation of IL-6 production in the cancer cells and the effect of the IL-6 on the bone microenvironment and the vicious cycle. This effect could not be achieved in the murine setting described in the experiments above, as the humanized antibody does not interact with the murine IL-6R and thus cannot inhibit the effect of tumour produced IL-6 on the murine bone microenvironment.

5 CONCLUSION

Bone is known to be the preferred metastatic site of breast cancer cells. The majority of the occurring lesions is osteolytic even though mixed lesions occur in 20 % of the cases. After the occurrence of bone metastasis breast cancer becomes incurable and significantly reduces the patient's quality of life and life expectancy. Clinical data suggest that IL-6 producing tumours have more aggressive phenotypes, and are more malignant. Breast cancer patients with increased IL-6 serum levels show a poor clinical outcome and prognosis. The role of IL-6 within the breast cancer bone metastasis setting is, however, poorly understood and it is thus important to find out the role of IL-6 within this setting.

Firstly, it is important to elucidate whether the reduction of IL-6 production in IL-6 producing breast cancer cells leads to reduced malignancy and growth of breast cancer cells *in vitro*. Secondly, it will be interesting to see whether reduction of the IL-6 production leads to reduced breast cancer cell growth in the bone micro-environment *in vivo*. If that is the case, it will thirdly be intriguing to find out whether IL-6 reduction has any effect on tumour growth in other sites and the bone.

The general aim of this research project was to better understand the role of IL-6 in the development of osteolytic breast cancer metastasis to bone. The project consisted of an *in vitro* and an *in vivo* part.

In the *in vitro* part IL-6 production in MDA-MB-231 cells was knocked down through lentiviral transfection. To create a proper control, cells were also transfected with an empty viral vector to ensure that the transfection process itself did not affect the IL-6 expression in the investigated cell line. The result of the knockdown was assessed and confirmed by measuring IL-6 mRNA levels and IL-6 protein levels in the knockdown cells and the non-target cells (81).

The *in vivo* part of the project consisted of 3 parts all which were directed towards assessing IL-6 deficient tumour growth in mice. Firstly, immune-deficient balb/c nu/nu mice were injected intratibially with the IL-6 depleted breast cancer cells and the tumour growth was monitored weekly by X-Ray measurements. The X-Ray measurements revealed reduced tumour growth of the knockdown cells in the bone microenvironment as compared to non-target cells. After 3 weeks the animals were sacrificed, the harvested tissue was subjected to μ-CT and histological analysis which confirmed the reduction of tumour growth in the animals injected with the IL-6 deficient cells.

Secondly, we assessed the effect of the IL-6 knockdown in another environment, namely the mammary fat-pad of nude mice. Two groups of nude mice received intra-mammary injections with one group receiving knockdown cells, the other non-target cells. The experiment was conducted for 16 days and the tumour growth was assessed with callipers. We did not see a difference in the growth of the two cell entities and thus concluded that IL-6 exerts its tumour promoting properties through interacting with the bone microenvironment and enhancement of the vicious cycle.

Thirdly, two groups of nude mice were injected with MDA-MB-231 parental cells and one of the groups received a treatment with the humanized IL-6R antibody Tocilizumab. Murine IL-6 is not able to interact with the human IL-6R on the tumour cell surface, so the only IL-6 capable of this interaction was the IL-6 produced by the tumour cells in an autocrine and paracrine fashion. This setup gave us the possibility to look at the autocrine and paracrine effect tumour-produced IL-6 has on breast cancer cell growth in bone. The experiment was again conducted for 3 weeks with weekly X-ray measurements performed and we also saw that the Tocilizumab treated animals exhibited reduced tumour growth. From this result we concluded that tumour produced IL-6 enhances tumour growth in the bone microenvironment in an autocrine and paracrine manner.

These findings may have clinical implications because the humanized IL-6R antibody Tocilizumab is now available and approved by the FDA. It could be administered as an adjuvant therapy to patients with advanced IL-6 producing breast cancers with bone metastasis and improve the quality of life of these patients and their disease outcome.

Further research needs be done with the engineered knockdown cells that might show a decreased homing of breast cancer cells to bone or decreased multi drug resistance. It is also very important to find out whether the administration of the IL-6R antibodies can reduce the occurrence of bone metastasis in IL-6 sufficient breast cancer and/or reduce drug resistance.

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Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

PUBLIKATIONEN

1) - “The role of tumour derived interleukin 6 in a murine model of breast cancer bone metastasis” Zheng Y, Mikuscheva A, Zhou H, Börnert K, Buttgereit F, Dunstan CR and Seibel MJ
2) - “Treatment with interleukin-6 receptor antibodies inhibits breast cancer growth in a murine model of bone metastasis“ Börnert K, Zheng Y, Zhou H, Mikuscheva A, Buttgereit F, Dunstan CR and Seibel MJ
3) - “RANKL Enhances Tumor-derived IL-6 Expression in Breast Cancer Metastasis in Bone” Zheng Y, Zhou, H., Fong-Yee, C, Mikuscheva, A., Buttgereit, F., Seibel, MJ, Dunstan, C.. J Bone MinerRes; 2009.

SELBSTSTÄNDIGKEITSERKLÄRUNG

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