Establishment of *in vitro* breast cancer bone metastasis model using an artificial microenvironment

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Abstract

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Advanced breast cancer frequently metastasises to the bone, causing considerable morbidity to the patients. Survival rate after detection of bone metastases is poor, and options to treat bone metastases are insufficient. The bone microenvironment provides a fertile ground for the breast cancer cells to migrate and populate to. Tumour cells and the microenvironment have complicated and dynamic interactions that induce altered activity and phenotype in tumour cells. Novel preclinical models are needed in cancer drug development for efficient selection process of drug candidates.

The aims of this thesis study were to establish an *in vitro* artificial bone microenvironment and to investigate possible altered sensitivity of metastatic breast cancer cells to cytotoxic agents in the formed microenvironment.

The artificial bone microenvironment was established by culturing MC3T3-E1 cells with the bioactive glass. Osteoblast differentiation was verified with alkaline phosphatase staining and mineralisation capability with von Kossa staining. Altered sensitivity of breast cancer cells to cytotoxic drugs was studied by incorporating 4T1-*Luc2* and using a luciferase reporter assay to evaluate cell viability after treatment.

The results of this thesis study showed that osteoblasts cultured together with S5P34 bioactive glass formed a functional microenvironment and osteoblast were able to differentiate and secrete mineralised matrix in it. An interesting finding was that 4T1-*Luc2* cells had increased sensitivity to 5-fluorouracil when cultured in artificial microenvironment when compared to cultures solely on plastic. The same effect wasn't observed with doxorubicin. The artificial bone microenvironment model proved attractive possibility and development of complex metastasis models should be continued.

Key words: breast cancer, bone metastasis, bone microenvironment model

Tiivistelmä

TURUN YLIOPISTO
Biolääketieteen laitos, Lääketieteellinen tiedekunta
SMITH, SARAH: Rintasyövän luustoetäpesäkkeen mallin luonti käyttäen keinotekoista mikroympäristöä
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Rintasyöpä on naisten yleisin syöpä ja pitkälle edetessään se muodostaa usein etäpesäkkeitä luuhun. Etäpesäkkeet luussa lisäävät sekä potilaiden oireita että kuolleisuutta, ja niiden hoitomahdollisuudet ovat rajalliset. Rintasyöpäsolut hakeutuvat usein luuhun, sillä luun mikroympäristö tarjoaa suotuisan ympäristön syöpäsolujen asettumiselle. kasvaa. Luun mikroympäristö ja syöpäsolut ovat jatkuvassa ja aktiivisessa vuorovaikutuksessa, joka johtaa muutoksiin syöpäsolujen toiminnoissa ja fenotyypissä. Syöpälääkkeiden kehityksen tehostamiseksi tulee kehittää menetelmiä, jotka mallintavat vuorovaikutusta mikroympäristön ja syöpäsolujen välillä.

Tämän Pro gradu -tutkielman tavoitteena oli luoda keinotekoinen luukudoksen mikroympäristö, käyttäen bioaktiivista lasia sekä osteoblastisoluja. Toisena tavoitteena oli selvittää, muuttuuko rintasyöpäsolujen herkkyys syöpälääkkeille, kun niitä kasvatetaan keinotekoisessa luukudosta muistuttavassa mikroympäristössä verrattuna syöpäsolujen kasvattamiseen yksin muovipohjalla.

Keinotekoinen mikroympäristö saatiin aikaan viljelemällä MC3T3-E1-osteoblasteja yhdessä S5P34-bioaktiivisen lasin kanssa. Osteoblastien erilaistuminen varmistettiin alkaliininen fosfataasi-värjäyksellä ja solujen toiminnallisuus tarkistettiin von Kossa-värjäyksellä, jolla voidaan erottaa mineraaliesiintymiä. Syöpäsolujen muuntunutta herkkyyttä doksorubisiini- sekä 5-fluorourasiili-lääkeaineille tutkittiin istuttamalla 4T1-*Luc2*-rintasyöpäsolut keinotekoiseen luuston mikroympäristö-soluviljelmään. Lääkeaineiden vaikutusta rintasyöpäsolujen elinkelpoisuuteen määritettiin lusiferaasi-mittauksilla.

Tämän tutkielman tulokset vahvistivat, että osteoblasti-solujen viljely yhdessä S5P34-bioaktiivisen lasin kanssa muodostaa toiminnallisen luuston mikroympäristön, jossa osteoblastit kykenevät erilaistumaan ja tuottamaan mineralisoituvaa luumatriisia. Kiinnostava löytö tutkielmassa oli rintasyöpäsolujen herkistyminen 5-fluorourasiilin vaikutukselle keinotekoisessa luuston mikroympäristössä, verrattuna syöpäsolujen viljelyyn yksinomaan muovilla. Samanlaista vaikutusta ei todettu doksorubisiinilla. Tämä luukudosta muistuttava mikroympäristömalli todettiin toimivaksi, ja vastaavien etäpesäkemallien kehittämistä tulisi jatkaa syöpälääkekehityksen edistämiseksi.

Avainsanat: rintasyöpä, etäpesäke, luuston mikroympäristö-malli

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1. Introduction

1.1. Breast cancer and bone metastasis

Breast cancer is the most common malignancy in women globally, accounting for 25% of newly diagnosed cancer cases. Breast cancer is also the leading cause of cancer-related deaths among women. (American Cancer Society, 2018.) Bone is the primary site of metastasis for several cancers and is most frequent in breast and prostate cancers in particular. Metastasis in the bone occur up to in 70% of the patients with an advanced breast cancer. Metastasis in the bone cause considerable morbidity to the patients that is also referred as skeletal related events (SREs). SREs include severe pain, pathologic fractures, spinal cord compression, impaired mobility and hypercalcemia. Furthermore, tumours metastasised in the bone greatly reduces patient survival, the five-year survival rate being only 20% in breast cancer patients after discovering bone metastasis. (Roodman, 2004.)

Precise numbers of bone metastasis prevalence are difficult acquire but estimations can be made. By estimation, approximately 350,000 people with bone metastases die each year in the United States. (Mundy, 2002.) More specific estimations have been made in a cohort study conducted in UK that used a database containing detailed information of over 7000 women with invasive stage I–III breast cancer, and women with an initial diagnosis of stage IV disease with visceral metastases but without bone metastases in Guy's Hospital, London. Of these women, 22% developed bone metastases during a mean follow up period of 8.4 years after first diagnosis of breast cancer. (Harries et al., 2014.) Another study at a single cancer centre in Canada that evaluated the incidence of bone metastases in metastatic breast cancer patients showed that of 264 patients 73% had skeletal metastases. The median time to develop bone metastases from primary breast cancer was 23.1 months and the median overall survival from the diagnosis of metastatic bone disease was 40 months. (Kuchuk et al., 2013.)

The consequences of bone metastasis to the patients are severe due to the decreased quality of life and the poor survival rate after diagnosis in addition to the loss of functionality that is caused by the SREs. Despite the recent progress in metastatic breast cancer treatment i.e. endocrine therapy and targeted therapies, no treatment is yet able to prevent skeletal metastases or stop the disease progression. The severely diminished quality of life and survival of the patients with bone metastases calls for development of

new therapeutic options. To achieve this, improved methods of drug development and novel systems to study bone metastatic progression are needed. (Liang et al., 2020.)

1.2. Bone microenvironment

Bone is a highly dynamic tissue that is constantly remodelled by the activity of bone cells. Bone is composed of bone cells and organic matrix of mainly collagenous proteins and inorganic mineralised extracellular matrix (ECM). Bone remodelling eliminates old and damaged bone and is followed by formation of new bone tissue. Healthy bone and remodelling are important in maintaining mechanical support and homeostasis in the body and it depends on several local and systemic factors such as hormones, cytokines, chemokines and biomechanical stimulation. Bone plays a central role in the mineral homeostasis and regulates the amount of calcium in the body. (Bussard et al., 2008.)

1.2.1. Osteoblasts

Osteoblast are mononucleated, morphologically cuboidal and polarised cells that reside along the surfaces of the bone. They are responsible for the synthesis and mineralisation of ECM of the bone. Osteoblasts arise from the mesenchymal stem cells and the differentiation process is tightly mediated by numerous factors at distinct time points. The key transcriptional factor of osteoblast differentiation is the Runt-related transcription factor 2 (Runx2, also known as Cbfa1). Runx2 drives the differentiation of mesenchymal progenitor cells to preosteoblastic cells by inhibiting the differentiation direction towards other cells types such as adipocytes. (Long, 2011.) Deletion of Runx2 gene in mice results in lack of mineralised bone due to defective osteoblast differentiation, demonstrating that the expression Runx2 is critical for normal bone function (Komori et al., 1997). Another central transcription factor Osterix direct the preosteoblasts to become committed to the osteoblastic phenotype and it is one of the downstream effects of Runx2 (Harada and Rodan, 2003; Nakashima et al., 2002.). Both transcription factors Runx2 and Osterix are regulated by several signalling pathways, such as osteoblast differentiation inhibiting Notch signalling, differentiation promoting Wnt-signalling and Bone morphogenetic protein (BMP) signalling that stimulates osteoblast differentiation, BMPs also regulate the function of mature osteoblasts. (Long, 2011.)

The main function of mature osteoblasts is the formation of new bone matrix. Osteoblasts secrete collagen and other matrix proteins such as osteocalcin and osteonectin outside the cell. The resulting, still unmineralised product is called osteoid. Precipitation and

accumulation of calcium phosphate ions occurs within the collagenous matrix of the osteoid, subsequently forming hydroxyapatite in a process that lasts several days. The non-collagenous proteins influence the mineralisation process as well, for example, osteocalcin is involved in binding the calcium and osteonectin function in linking the collagen and mineral component to each other. Osteoid also contains inhibitory factors that regulate the bone formation process. One of the inhibitory factors is pyrophosphate which prevents the inorganic phosphate from crystallising with calcium in order to form hydroxyapatite. (Young et al., 2014.) In addition to matrix proteins, osteoblasts also deposit membrane-bound matrix vesicles that contain aggregates of calcium and build-up of hydroxyapatite that is consequently released into the sites of developing matrix. Matrix vesicles are enriched in proteins and enzymes that take part in the mineralisation process of osteoid. (Vimalraj, 2020.) One such enzyme is alkaline phosphatase (ALP) that cleaves the pyrophosphatase and therefore actively neutralises the inhibitory effect of pyrophosphatase, allowing mineralisation process to continue. ALP is exhibited in proliferating osteoblasts and it is significantly enhanced also during *in vitro* bone formation. Detecting ALP using methods of histochemical staining are used in osteoblast characterisation and differentiation, since after bone matrix mineralisation ALP activity can no longer be detected. (Roach, 1999.)

1.2.2. Osteoclasts

Osteoclasts are multinucleated and terminally differentiated cells derived from the monocyte-macrophage lineage of hematopoietic stem cells. Differentiation of osteoclasts is controlled by locally produced cytokines and systemic hormones. One of these factors, macrophage colony-stimulating factor binds to the receptor on osteoclast precursors and stimulate their proliferation and survival. (Kodama et al., 1991.) Another factor regulating the development of osteoclasts is the receptor activator of nuclear factor κB ligand (RANKL), a potent inducer of osteoclast formation that is expressed by osteoblasts. RANKL binds to is receptor RANK (receptor activator of nuclear factor κB) and the the RANK/RANKL interactions have numerous downstream effects it is largely regulated by osteoblasts. (Boyce and Xing, 2008.) Osteoclasts are phagocytic cells overseeing bone resorption by dissolving the mineral component of the bone. Osteoclasts form a tightly sealed zone in which they secrete proteases and acid. Proteolytic enzymes actively degrade matrix proteins and HCL dissolves the inorganic hydroxyapatite of the bone. (Tzelepi et al., 2009.)

1.2.3. Osteocytes

Osteocytes are the most common and long-lived cells in the bone tissue, representing up to 95% of the total bone cells. They differentiate from osteoblasts when a sub-population of osteoblasts become surrounded by the bone matrix they produce and subsequently undergo morphological and functional changes. Osteocytes are thus buried in the mineralised bone matrix and their protein synthesis and secretion is reduced and the cells decrease in size. Regardless the phenotypic changes and isolated location, osteocytes significantly contribute in bone metabolic activities. Osteocytes are mechanosensitive and respond to mechanical stimulus in the bone. They also react to changes in ion concentration in the environment and alter their subsequent signalling pathways in order to regulate bone the bone homeostasis. (Tzelepi et al., 2009.) Osteocytes secrete several signalling factors such as RANKL and osteoprotegerin (OPG) that regulate the functions of osteoblasts and osteoclasts and thus influence the activities bone resorption (Ina Kramer et al., 2010).

1.2.4. Extracellular matrix

The extracellular bone matrix is composed of approximately 30% organic matrix and 70% inorganic minerals. The organic matrix of bone is made up 90% of collagenous proteins, that is primarily type 1 collagen, and the remainder consists of non-collagenous proteins and proteoglycans including osteocalcin, osteonectin and sialoproteins and growth factors. The inorganic mineral component mainly consists of calcium and phosphate but other salts as well, for example potassium, bicarbonate, sodium and magnesium. The mineral component consists mainly of calcium and phosphate which are arranged in the form of hydroxyapatite crystals, often presented by the formula $Ca_{10}(PO_4)_6(OH)_2$. Together the hydroxyapatite and organic matrix of collagen and other proteins form a complex and an organised scaffold that is responsible for the resistance and rigidity of the bone. The bone ECM does not only provide mechanical support but plays an important homeostatic role and participates to the bone remodelling. (Young et al., 2014.)

1.3. Metastatic process of breast cancer cells to the bone

Metastasis to the bone is a dynamic, multi-step process in which the cancer cells undergo significant phenotypic and genotypic changes (Figure 1). The process begins in the primary tumour when the cancer cells lose their epithelial features and disconnect from their surroundings, enabling the invasion of the primary tumour cells to the surrounding

tissues. The tumour cells lose their adhesive and polarity features and acquire invasive and migratory properties, increased motility for example. This phenomenon is called the Epithelial-Mesenchymal transition (EMT). Cells undergoing EMT excrete proteolytic signals that permit them to intravasate into the blood stream and become circulating tumour cells (CTCs). (Hanahan and Weinberg, 2011.) Previous research shows, that primary tumours with metastatic capabilities can be differentiated from non-metastatic ones. A study comparing gene expression profiles of primary tumours of breast cancer patients with positive or negative findings of bone metastases showed distinctively two expression profile groups matching their metastatic status demonstrating that bone metastasis is a selective process that requires a specific molecular signature of the cancer cells. (Woelfle et al., 2003.) It also highlights that metastatic cells diverge greatly from the primary tumour cells.

Once the CTCs have entered the circulation, most CTCs are destructed by the immune cells and natural attrition. Approximately nnly under 1% survive and successfully migrate to new sites. Successful CTCs invade, or extravasate, into the bone marrow cavities becoming disseminating tumour cells (DTCs). A metastatic tumour cell niche is therefore created and surviving DTCs interact with the bone microenvironment, entering either immediate proliferation and populate in the bone marrow or go into dormancy. (Qiao and Tang, 2018.) Dormancy is a stage in which the tumour cells have homed and resided in the bone marrow but entered a dormant phase and appear to remain in a non-proliferative and non-metabolic stage (Aguirre-Ghiso, 2007). Dormant cells can be resistant to chemotherapies, signifying that patients may remain asymptomatic and seemingly recovered after treatments, but relapses in breast cancer may occur even after several years after treatment or surgical resection of the primary tumour (Braun et al., 2005). The bone microenvironment greatly determines the course of DTCs and the reciprocal interactions that may drive the tumour cells to either proliferation or dormancy are extensively reviewed by Aguirre-Ghiso (2007).

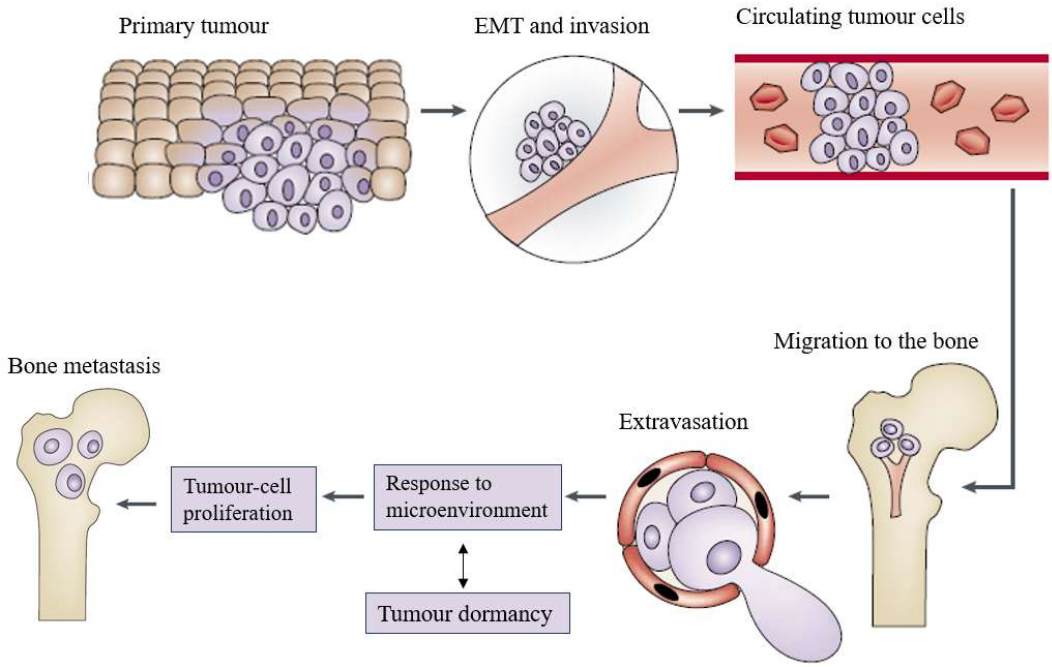

Figure 1. Metastatic process of breast cancer cells from primary breast tumour to bone metastases. Metastasis to the bone is a dynamic process, in which the tumour cells are constantly challenged. Tumour cells that are capable of undergoing Epithelial-Mesenchymal transition (EMT) can invade to the surrounding tissues and into the circulation. Circulating tumour cells that survive in the circulation migrate and extravasate into the bone. The metastatic tumour cells interact with the bone microenvironment and may go into dormancy or directly proliferate and populate in the environment that is supportive of tumour growth. (Adapted from Mundy, 2002)

1.4. Osteotropism of breast cancer

Metastasis to the bone is not a random occurrence. Stephen Paget's well-known hypothesis proposes that different organs provide optimal growth conditions to specific cancers, and the bone is considered a favourable site for metastatic breast cancer cells for several reasons. For instance, the blood flow to the highly vascularised bone marrow transport the metastatic cells there and assist in the establishment of metastases but also the specific properties of the local bone microenvironment determine the selectivity of bone. Bone has a large repository of a variety of growths factors and cytokines that are released during normal bone resorption and facilitate the growth of metastatic cells. Then again, breast cancer cells secrete molecules that modulate the activity of osteoblasts and osteoclasts. (Mundy, 2002.)

Osteotropism of breast cancer has been demonstrated to be at least partly caused by certain chemokines, a group of signalling proteins binding to G-protein-coupled receptors

that have a well-established role in leukocyte migration and adhesion into bone marrow. An example of one of the most well studied chemokines in breast cancer cell migration and invasion is CXC chemokine ligand 12 (CXCL12 also known as stromal cell-derived factor, SDF-1). High levels of CXCL12 are released only in certain organs such as the bone marrow and it is highly expressed by osteoblasts. (Müller et al., 2001.) Its receptor pair CXC chemokine receptor 4 (CXCR4) is expressed by breast cancer cell surfaces and CXCR4 has been shown to be expressed at elevated levels in a sub-population of breast cancer cells that specifically home to bone, enlightening one of the mechanisms of how breast cancer cells favour forming metastases to bone (Kang et al., 2003).

1.5. Bone microenvironment and tumour cell interactions

The ability of breast cancer cells to grow and populate in the bone is greatly contributed by the molecular interactions between tumour cells and the microenvironment (Figure 2). Exact mechanisms of tumour-microenvironment crosstalk are still unknown, although several factors have been identified. (Hiraga, 2019.) The most abundant growth factor family that is restored in the bone are insulin-like growth factors (IGFs). IGFs are associated with numerous cancer types and are implicated to have a role in the development, progression and aggressiveness of cancers, breast cancer included. For example, breast cancers express increased levels of IGF type I (IGF-1) receptors (IGFIR) and when initiated by bound IGF, several pathways are activated that promote malignant behaviour of the cancer cells. (Hiraga et al., 2012.) Activation of IGFIR has also been directly linked to increased cancer growth and metastasis to bone *in vivo* (van Golen et al., 2006).

One of the most recognised interactions between metastatic tumour cells and the bone microenvironment is the excessive production of parathyroid hormone-related protein (PTHrP) in the tumour cells. PTHrP activates RANKL signalling in osteoblasts which binds to RANK in osteoclasts, activating osteoclasts precursors and enhancing bone resorption. Following the increased amount of bone resorption, more growth factors i.e. TGF-β, IGF and BMPs are released from the bone reservoirs and promote proliferation of cancer cells and increases production of PTHrP. This imbalance in bone resorption is referred as "vicious cycle" of continuous tumour and bone cell activation. (Roodman, 2004.) Several studies support this concept. For example, PTHrP expression is higher in breast cancer cells that have metastasised to bone compared to non-skeletal metastases (Powell et al., 1991) and TGF-β increase the production of PTHrP (Yin et al., 1999).

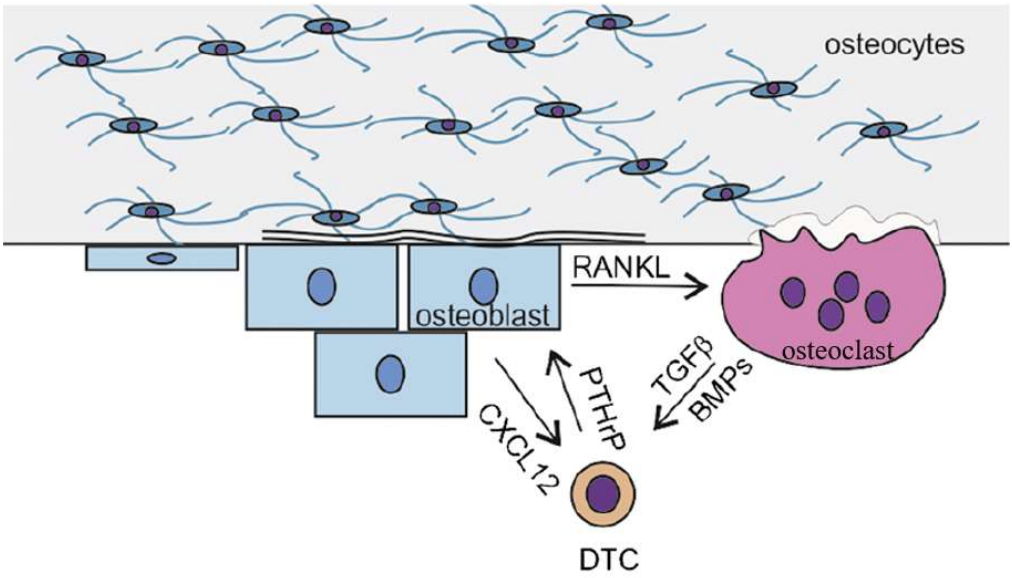

Figure 2. Cell interactions in the Metastatic Bone Microenvironment. Multiple cell types are represented in the metastatic bone microenvironment. The microenvironment is populated by the metastatic disseminated tumour cells (DTCs) from the primary breast tumour, which then interact with the bone cells. Osteoblasts secrete CXCL12 cytokine that attracts the metastatic tumour cells. Bone cells also interact with the DTCs via several other signalling mechanisms such as RANKL, PTHrP, TGFβ and BMPs. (Adapted from Heino and Määttä, 2018.)

The bone microenvironment interacts with cancer cells also by physical stimuli in addition to the cellular and molecular interactions. Cancer cells respond to mechanical stress, hypoxia and the acidic environment in the bone microenvironment. (Fournier et al., 2015.) Breast cancer cells are affected by the hypoxic conditions in the bone

microenvironment. The cell responses to hypoxia are primarily mediated by the hypoxia-inducible factors (HIFs). Under hypoxic conditions, the subunits hypoxia-inducible factor 1α (HIF1α) and the hypoxia-inducible factor 1β (HIF1β) are stabilised and heterodimerised causing changes in the transcription of target genes. (Hiraga, 2018.) Overexpression of HIF1α has been recognised in metastatic breast cancer (Zhong et al., 1999) and the knockdown of HIF1α in MDA-MB-231 breast cancer cells inhibited the formation of bone metastases in a mouse model and improved their survival (Dunn et al., 2009). Hypoxic conditions upregulate enzymes of the glycolytic pathway via HIFs in tumour cells and thus intensify the glycolytic energy metabolism switch that is also referred as Warburg's effect, an abnormal characteristic of cancer cells using glycolysis as a main energy metabolism source despite its relatively poor efficiency (Hanahan and Weinberg, 2011).

Breast cancer cells also react to the mechanical environment and stiffness caused by the mineralised matrix in the bone microenvironment. Cancer cells respond to the increased rigidity in their surroundings via the transmembrane protein integrin αvβ3 which is found to be upregulated in bone metastasising tumours compared to normal tissues. (Kwakwa and Sterling, 2017.) One study has demonstrated that osteotropic MDA-MB-231 breast cancer cells expressed greater levels of integrin αvβ3 compared to their parental cells (Pecheur et al., 2002). Integrin αvβ3 has shown to cause modifications in the gene expression of metastatic tumour cells in response to the matrix rigidity. For example, a group of investigators demonstrated that integrin αvβ3 upregulated a signalling cascade that induced an increased PTHrP expression, which is known to drive the "vicious cycle" of metastatic cancer cell growth. (Page et al., 2015.)

1.6. Modelling bone metastases

Cell-based modelling is a well-established method of cancer research. However, models for investigating bone metastases and how to better illustrate the role of the microenvironment in them needs still further development and calls for using preclinical models and testing that they reliably predict the therapeutic efficacy of candidate agents. (Bhadriraju and Chen, 2002.)

1.6.1. *In vivo* models

One of the traditional methods to create a bone metastasis in a *in vivo* model is the orthotopic injection of breast cancer cells into the mammary fat pad of immunodeficient

mice. This method has potential of modelling the whole metastatic processes from the primary tumour growth to the development of metastasis at a distant site. Other frequently used methods of *in vivo* models are intraosseous and intracardiac murine models. Intraosseous models are created by implanting tumour cells directly in the bone, into the tibia or femur of a mouse, causing metastasis to arise rapidly. The intraosseous model bears a resemblance only to the final stages of bone metastasis and thus prevents the possibility of studying homing of tumour cells in the bone and the early interactions between the microenvironment and tumour cells such as extravasation and dormancy. Intracardiac models are created by injecting tumour cells into the left ventricle of mice and they generate bone metastasis at a high frequency, recapitulating the extravasation from circulation to the bone tissue. (Jinnah et al., 2018.)

Using models where tumour cells are introduced into experimental animals, mainly mice, present a major limitation of needing to use immunocompromised mice. They lack significant immunological factors, which play a significant role in the process of metastatic development and many key interactions are thus missing. (Jinnah et al., 2018.)

Efforts in improving *in vivo* models to better recapitulate the human bone microenvironment have been made, resulting in humanised mice models. These models utilise either human or synthetic bone implants in mice in addition to implanting human tumour cells, the objective being that the bone implant serves as target for the metastatic process. Engineered or synthetic constructs have emerged as a tempting choice due to their reproducibility and better availability than human bone implants. (Jinnah et al., 2018.) However, a great limitation of humanised *in vivo* models presents in low or variable frequency of the metastasis to the bone implant and the length of time before occurrence of metastasis, which may take often up to 5-6 months. Overall, the low frequency of metastasis in animal models and the lack of complete immunosystem make *in vivo* models mostly unsuitable for studying metastases and effectivity of treatments. (Holen et al., 2015.)

In addition, using *in vivo* models in drug discovery have been found to be non-reliable as evaluation for drug efficacy and animal models have largely recognised limitations, the most predominant ones being their incompatibility to high-throughput drug screening because of high costs, time-consuming testing, and ethical aspects. (Sharma et al., 2010.)

1.6.2. Two-dimensional (2D) cell culture models

Along with the increasing understanding of the importance of the microenvironment to tumour development, the relevance of classical two-dimensional (2D) monolayer cell cultures has started to be questioned (Sharma et al., 2010). Whether 2D cell cultures represent the cell activities and phenotype sufficiently in order to have any relevance with the *in vivo* or clinical circumstances and whether they should be thus used in screening for suitable drug candidates is arguable. 2D cultures on artificial plastic surfaces lack the ECM, leading cells to lose distinctive features such as the correct spatial orientation and population heterogeneity. Essential signalling cues for differentiation, proliferation, polarisation, and other functions are therefore lost. Consequently, this loss is of extreme disadvantage in bone metastasis modelling since the ECM has fundamental influence on the tumour cell signalling and growth. Essential biological responses to pharmacological agents may not be physiologically reproduced in 2D cultures and it may therefore hinder the evaluation of drug efficiency and therefore their use in drug discovery. (Breslin and O'Driscoll, 2013.)

1.6.3. Three-dimensional (3D) cell culture models

Three-dimensional (3D) culture models represent a more reliable approach for studying interactions within tumour cells and microenvironments. The gap between 2D cultures and *in vivo* studies are proposed to decrease with 3D culture models. 3D cultures differ from 2D culture in that the cells are promoted to form 3D spheroids of cells and by introducing matrices instead of growing cells in a monolayer. 3D models have gained popularity in cancer drug development due to them being more predictive of *in vivo* events than 2D cell cultures. (Breslin and O'Driscoll, 2013.) A study comparing a panel of malignant and non-malignant breast cell lines cultures in 2D monolayer cultures and 3D cultures revealed significant differences in gene expression in relation to the culturing conditions, supporting that 3D cell cultures alter cellular activity and enforcing the concept that cells behave differently depending on their microenvironment (Kenny et al., 2007).

3D modelling systems better recapitulate the multicellular structure and interactions of cell-cell and cell-matrix cross talk and lead to spatially and architecturally applicable similarity to *in vivo* tissue. Thereby, 3D cultures provide relevant biological properties to the tumour cells. There are several different approaches to construct 3D cell culture

models of bone metastases. Multicellular tumour spheroids assemble cell aggregates in bioreactors that have characteristics of solid tumour such as necrotic core and uneven gradients of signalling molecules. They may consist only of tumour cells o or include for example stromal cells also. (Qiao and Tang, 2018.) However, the lack of ECM components is one of the most often stated problems of these systems since the cell-ECM interactions and spatial architecture of the ECM contributes to the tumour cell responses and signalling pathways as also previously described in chapter 1.5. The ECM can alter the performance of drugs via several ways. The composition, organisation, and stiffness of ECM (i.e. high collagen composition) may alter drug efficacy. Also, cell-ECM adhesion molecules such integrins activate survival promoting signalling in tumour cells supporting chemoresistance against cytotoxic drugs. (Holle et al., 2016.)

This disadvantage has been tackled with introducing artificial components mimicking ECM to the *in vitro* 3D cultures. Matrigel is a commercially available matrix that has incorporated parts of basement membrane proteins. Cells grow embedded in or on top of the matrix and are able to generate spheroids that have a stromal support structure. There are multiple different kind of matrices available for spheroid generation in ECM; yet even this model fails to recreate the complex cancer-mineralised matrix interphase. (Breslin and O'Driscoll, 2013.) Scaffolds are an alternative method of 3D culturing. They are based on either natural materials or synthetic constructs and are made of wide range of materials and mechanical characteristics. Synthetic scaffolds are often processed to form hydrogels that are commonly composed of collagen or laminin. Hydrogels are porous materials and when cells are seeded into the scaffold, they are able to migrate between the scaffold fibres and form 3D structures. Hydrogels that integrate molecules found in the ECM are able to exchange signalling cues closely mimicking the signalling experienced in *in vivo*. (Breslin and O'Driscoll, 2013.) Natural scaffolds can be collagen-based, or osteoblast derived to name some examples. Mineralised matrix of osteoblasts has been used in cell cultures and reported to support metastatic breast cancer cell progression. However, fully natural scaffolds are difficult manufacture and have unreliable durability. They should also be cost efficient and feasible for sufficient throughput for high capacity screening. (Qiao and Tang, 2018.)

1.7. Bioactive glass and their properties and applications

Bioactive glasses first described by L. Hench in 1971 are synthetic silica-based materials that can form chemical bonds to the bone. Bioactive materials are defined as materials

that stimulate a biological response from the body at the interface of the material and the tissue. Bioactive glasses are osteostimulative, meaning that they have properties that enable osteoblast cell recruitment or activation, ultimately producing new bone tissue. Bioactive glasses are also osteoconductive, meaning that they introduce the possibility for bone to grow along the material or that they provide a scaffold for bone formation. There are various compositions of bioactive glasses. The first-generation bioactive glasses such as Bioglass® 45S5 and S53P4 are composed of SiO_2, Na_2O, CaO and P_2O_5. The activity rates of bioactive glasses differ by their composition and is mostly reliant on the proportion of SiO_2. Novel bioactive glasses contain also K_2O-MgO and they can be manufactured in several different forms, overcoming the issues of first-generation glasses, such as crystallisation of the glass in high temperatures. (Välimäki and Aro, 2006.)

Activity of bioactive glasses are based in their ability to stimulate biological responses from the body at the interface of the material and the tissue. A reaction on the glass surface is initiated after contact with body fluids which promotes a rapid release of sodium, silica, calcium, and phosphate ions. This results in a local increase of pH, that alkalinise the surroundings. After the release of the ions, a silica-rich gel layer is formed on the bioactive glass surface. Formation of the silica layer on the surface is a critical step for the bonding of the bone as it acts as a template for calcium phosphate precipitation. The calcium phosphate layer then crystallise to hydroxyapatite and directs new bone formation by activating osteoblasts. These surface reactions do not only promote the formation of new bone, but they also contain antibacterial properties and have angiogenic potential. (van Gestel, N. A. P. et al., 2015.)

S53P4 bioactive glass is used in several clinical applications such as bone grafts in craniofacial reconstructions and in treatment of osteomyelitis, an infection of bone and bone marrow in which the antimicrobial properties of S53P4 are utilised. S53P4 bioactive glass is described to degrade slowly in the body, but it is mostly found to be relatively stable. Remnants of glass granules may be found up until 14 years after implantation (Peltola et al., 2006; Lindfors et al., 2010.)

Bioactive glasses have shown to have properties that aid growth and maturation of osteoblasts, but the mode of the actions that affect the cells are not quite clear. The most agreed osteostimulative effect of bioactive glasses are the surface reactions and the

formation of the silica layer. In a study investigating the stimulatory effects of bioactive glass showed that the released ions of the Bioglass 45S5 increased osteoblast cell proliferation. Interestingly, the research group also presented that the insulin-like growth factor II (IGF-2) gene was overexpressed, and the amount of secreted unbound IGF-2 was increased. (Xynos, Ioannis D. et al., 2000.)

The introduction of bioactive glass in preclinical cancer research is an interesting possibility. It is a well-characterised, homogenous and non-reactive material, that could mimic bone without invoking dramatic undesirable reactions.

1.8. Therapeutic treatment of bone metastatic breast cancer

Treatment of cancer patients with bone metastases aims to prevent further progression of the disease and prolong the survival of patients. They also aim to reduce the SREs and bone pain. Treatments can decrease the size or slow the growth of the metastases and therefore help manage the SREs and increase quality of life of the patients. Nevertheless, no treatment is curative or eliminate the metastatic growths. Treatment options for breast cancer patients with bone metastasis include bone targeting agents and cancer cell targeting agents. Bone targeting affect the bone microenvironment and are mostly indicated for osteoporosis while cancer targeting agents target the tumour cells themselves. (Harbeck and Gnant, 2017.)

1.8.1. Bone targeting agents against bone metastasis

Bisphosphonates are pyrophosphate analogues that bind strongly to the exposed hydroxyapatite in the bone and inhibit the osteoclast-mediated bone resorption. Bisphosphonates reduce the incidence of SREs such as hypercalcemia, pain and pathological fractures and they even reduce the formation of new metastases to the bone in breast cancer patients, though the mechanism of this effect remains unclear (Hortobagyi et al., 1996; Diel et al., 1998.) Another bone targeting agent used in bone metastasis treatment is denosumab, a human monoclonal antibody against RANKL. Denosumab inhibits RANKL and blocks the RANKL-RANK interaction, resulting in reduction of osteoclast activity and thus bone resorption. This mechanism aims to stop the “vicious cycle” driven by the breast cancer cells that leads to uncontrolled cell activity and growth. Using denosumab in bone metastasis treatment of breast cancer patients has shown to be successful in prevention of SREs, demonstrated as delays in the onset of

SREs compared to the treatment with the bisphosphonate agent zoledronic acid. (Alison T. Stopeck et al., 2010.)

1.8.2. Cytotoxic agents against bone metastases

Treatment and selection of agents for metastatic breast cancer treatment always requires an individual plan based on the specific tumour biology and previous treatment history of the patient. Traditional chemotherapeutics including anthracyclines (doxorubicin and epirubicin), taxanes (paclitaxel and docetaxel) and fluoropyrimidines (capecitabine and 5-fluorouracil) can be used as individual agents or in adjuvant therapies. (O'Shaughnessy, 2005.) Clinical trials using chemotherapeutic agents show initial response and improvements in disease control, but only small portion of patients achieve remission. (Alba et al., 2004; Greenberg et al., 1996.) After the introduction of biological agents such as trastuzumab, bevacizumab and pertuzumab that affect specific molecular targets in the tumour, improvements in overall survival of metastatic breast cancer patients has been observed. Biological agents are also used in combination with chemotherapy. (Harbeck and Gnant, 2017.) Clinical trials involving both chemotherapeutics and biological agents have demonstrated improved overall response and time to progression rates compared to chemotherapies alone in metastatic breast cancer patients (Slamon et al., 2001; Swain et al., 2015).

More recent treatment options are also in use of metastatic breast cancer treatment. Endocrine therapies that inhibit estrogen-promoted tumour growth such as aromatase inhibitors (anastrozole, exemestane, and letrozole) or tamoxifen, a selective estrogen receptor modulator that competitively blocks the receptor, are used often in initial treatment. Endocrine therapy is typically incorporated with a cyclin-dependent kinase 4/6 inhibitor (i.e. palbociclib, abemaciclib, or ribociclib). These lines of treatment are generally continued until the tumour becomes endocrine resistant and patients transition to chemotherapy treatments. Targeted inhibitors such as PARP inhibitors olaparib and talazoparib are used in patients with specific germline mutations. (Waks and Winer, 2019.)

Palliative treatment to treat bone pain that is caused by the localised metastases is performed by external radiotherapy. Radiation therapy inflict damage to both cancer cells and non-cancer cells via the ionising radiation that cause direct DNA damage. (Popovic et al., 2015.) Pain relief is usually rapid and treating the involved bone by local

radiotherapy have presented pain relief rates of 80-90% (Gaze et al., 1997). In addition to external radiotherapy, internal systemic radiotherapy using bone-seeking radioisotopes are used as palliative bone pain treatment. Three radionuclides, Strontium-89 hydrochloride, Rhenium-186 hydroxyethylidenediphosphonate and samarium-153 lexidronam are approved to treat metastatic bone pain. (Christensen and Petersen, 2011.)

Although there are several options for metastatic breast cancer treatment, therapeutic resistance of chemotherapeutic drugs and endocrine therapy remains a serious issue. Tumours may be intrinsically resistant, or response rates decline during multiple courses of treatment. (Waks and Winer, 2019.)

1.8.3. 5-fluorouracil

Chemotherapy agent 5-fluorouracil (5-FU) is widely used in cancer treatment, including breast cancer. It was first synthesised in 1957 and is a fluorinated pyrimidine and an analogue of uracil, one of the four bases found in RNA. (Heidelberger et al., 1957.) 5-FU acts as an antimetabolite, meaning that it interferes with the synthesis of DNA and RNA by substituting the normal building blocks required for DNA replication and transcription. 5-FU is typically used in combination therapies with other antitumor agents. 5-FU has been found effective in advanced breast cancer with the overall response rate varying from 29% to 54% and improving SRE related bone pain by 53%. (Cameron et al., 1994; Regazzoni et al., 1996.)

The antitumor effects of 5-FU are mediated by the inhibition of thymidylate synthase (TS). 5-FU is converted to three main metabolites in the cell, fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). FdUMP binds covalently to TS blocking its function and the binding of TS metabolites to DNA and RNA. This blocks the normal functions of RNA and DNA and ultimately ceases DNA synthesis and cell proliferation. TS is also a catalyser and limiting factor of thymidylate enzyme, which is required for DNA replication and repair. The loss of thymidylate eventually causes DNA strand breaks and cell death. The exact mechanism of the downstream effects causing the disruption in thymidylate synthesis is not quite clear but is thought to be caused by imbalances in the deoxynucleotides. Also, the misincorporation of 5-FU metabolite FdUTP in RNA cause disruptions on several levels of RNA processing. It inhibits the development of mature

ribosomal RNA and the post-transcriptional modifications of transferring RNA. (Longley et al., 2003.) 5-FU acts in the S-phase of cell cycle (Shah and Schwartz, 2001).

1.8.4. Doxorubicin

Doxorubicin is another common anticancer drug used in breast cancer treatment. Doxorubicin is grouped in anthracyclines, a group of drugs that interfere with the enzymes involved in DNA replication. Doxorubicin enters the cell by diffusing, and it forms a complex with the proteasomes in the cell cytoplasm. After repositioning the complex in the nucleus, doxorubicin relocates itself and binds to the DNA, intercalating between the base pairs in the DNA double helix. This inflicts breaks in the DNA that eventually cause arrest in cell proliferation. (Aubel-Sadron and Londos-Gagliardi, 1984.) Another cytotoxic mechanism of doxorubicin is mediated by the inhibition of topoisomerase II, an enzyme regulating the helical state of DNA by relaxing the supercoils. When the topoisomerase II is inhibited it ceases DNA replication and terminates cell proliferation. A third suggested mechanism of doxorubicin cytotoxic effects is the production of free radicals and reactive oxygen species (ROS) that cause damage to lipid membranes in the cells. (Giorgio Minotti et al., 2004.) It has been confirmed that doxorubicin induces a potent arrest in the growth or G-phase of cell cycle that is mediated by the p53 tumour suppressor protein response, ultimately causing cell cycle arrest and apoptosis (Attardi et al., 2004).

1.9. Inefficiency of Cancer Drug Development

By recent estimations of the drug development process succession rates, or the probability of success (POS), throughout all the development phases is 3.4% in cancer drugs while overall POS for all treatment indications (excluding oncology) is 23.4%. The POS of Phase 1 to Phase 2 in cancer drugs is 57.6% and overall (excluding oncology) it is 75.8%. These statistics demonstrate the importance of increasing the potential of cancer drug lead indications in early developmental phases for them to succeed in clinical development as well. (Wong et al., 2019.) Early identification of drug candidates which are ineffective and have high toxicity would improve the succession rate of new drugs and increase the efficacy of the whole drug development process and lower the costs of failed compounds. Resources could be directed early to the promising drug candidates. In order to overcome these issues and to develop better treatment strategies, improvement of cell-based *in vitro*

models and assays is needed so that screening and prioritising promising drug candidates would be successful. (Breslin and O'Driscoll, 2013.)

1.10. Aims of the study

The development of innovative preclinical systems that recapitulate the complex microenvironment of the breast cancer bone metastasis is critical for the development of novel treatments and for aiding in the decision making of advancing candidate drugs to later stages of the drug development pipeline. Many of the matrices and scaffolds used in 3D modelling lack the soluble signalling cues or the possibility of cells to adhere in the microenvironment, both extremely important in stimulating cell responses that are present in the biological bone microenvironment. (Breslin and O'Driscoll, 2013.) The introduction of bioactive glass in preclinical cancer research is an interesting possibility because it is a well-characterised, homogenous and non-reactive material, which mimics bone without invoking dramatic reactions. Previous research suggests that cells have altered function when cultured alone, deprived from the exterior contact with ECM and other cell types, which normally are present in a natural environment (Holle et al., 2016).

We therefore hypothesised that in a more biologically relevant growth environment where bioactive glass would simulate the ECM the breast cancer cells would react to pharmaceutical agents with an altered sensitivity compared to when the cultured alone. In this thesis study, we used an artificial bone microenvironment cell culture model to investigate whether breast cancer cells have an altered sensitivity to the cytotoxic agents', i.e. doxorubicin and 5-FU. The artificial bone microenvironment was established using a murine preosteoblastic cell line MC3T3-E1 and S5P34 bioactive glass granules. Thus, the two aims of this thesis project were:

I. Establish an artificial bone microenvironment modelling system using preosteoblastic cell line MC3T3-E1 and bioactive glass S5P34.

II. Study the possible altered sensitivity of 4T1-Luc2 cells to doxorubicin and 5-FU in the artificial bone microenvironment.

2. Results

2.1. Osteoblasts actively form matrix in the presence of bioactive glass particles

Differentiation and mineralisation capabilities of the preosteoblast cell line MC3T3-E1 were verified by ALP and von Kossa stainings when the cells were cultured together with the bioactive glass S53P4. Preosteoblasts were cultured in osteogenic culture media that induces osteoblast differentiation and ALP staining was conducted on the eight day of culturing. As can be seen from Figure 3A, some red-pink colouring of ALP staining is exhibited in the cell culture, indicating successful differentiation of preosteoblasts when cultured together with bioactive glass.

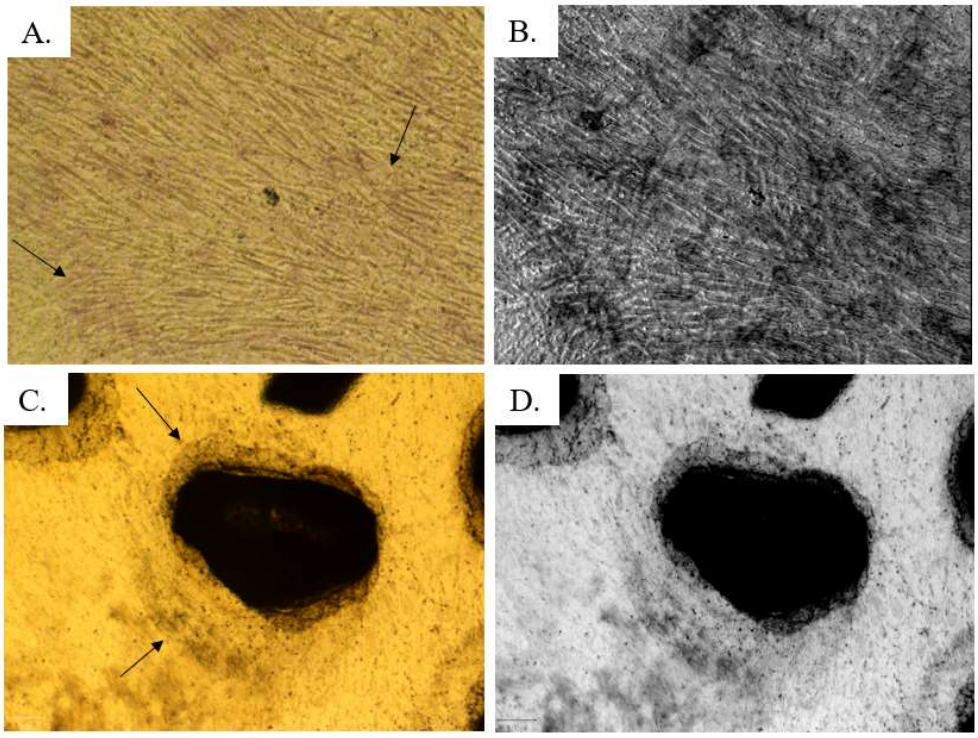

Figure 3. Alkaline Phosphatase and von Kossa staining of MC3T3-E1 osteoblasts cultured in the presence of S53P4 bioactive glass. A. Alkaline Phosphatase staining of fixed MC3T3-E1 preosteoblast cells when cultured together with S53P4 bioactive glass at day 8 of culture. Arrows indicate to pink staining. Image B. is an enhanced contrast image of A. C. von Kossa staining of fixed cells of MC3T3-E1 preosteoblasts cultured in the presence of S53P4 bioactive glass at day 21 of culture. A S53P4 granule is seen as the large dark bulk in the image. The arrows point to staining of calcium deposits and the matrix in the proximity of the glass granule. Image D. is an enhanced contrast image of B. Images are taken with 10x magnification.

Von Kossa staining on day 21 of cell culturing in same conditions exhibit the dark brown staining, representing the mineral deposits secreted by osteoblasts. The bioactive glass granule is visible as the large bulk in the middle of the image (Figure 3C-D). Calcium deposits demonstrate that the osteoblasts are functional and have successfully formed bone mineral *in vitro* bone. Also, in Figures 3C-D, a formed but unidentified matrix can be seen in the proximity of bioactive glass granule.

2.2. IC50 determination to evaluate cytotoxicity of 5-FU and doxorubicin in breast cancer cells

The cytotoxic effects of 5-FU and doxorubicin on 4T1-*Luc2* breast cancer cells were investigated using Alamar blue viability assay and by determining the IC50 values for the pharmaceutical agents. IC50 is the concentration of an inhibitor drug where the response to the drug is reduced by half, showing 50% of growth inhibition. IC50 values of 5-FU and doxorubicin were determined to evaluate the drug concentration range that cause cell death on the breast cancer cells when being cultured in normal conditions on solely plastic.

Table 1. IC50 values of 5-fluorouracil and doxorubicin determined by alamar blue viability assay at two different endpoints.

Endpoint	5-fluorouracil	Doxorubicin
48 h	2.8 mM	4.1 uM
72 h	3.7 mM	1.9 uM

The dose-response data of the pharmaceutical agents on breast cancer cells is presented in Figures 4 and 5. The decreasing fluorescence response of the viability assay when increasing the concentration of the drugs indicated dose-dependent toxicity caused to the 4T1-*Luc2* cells. The IC50 values of 5-FU in 4T1-*Luc2* breast cancer cells grown on plastic for 48h was 2,8 mM and grown for 72h the IC50 was 3,6 mM. For doxorubicin the IC50 value was 4,1 µM for the 48h endpoint and 1,9 µM for the 72h endpoint (Table 1). Thus, doxorubicin has a more potent toxic effect on 4T1-*Luc2* cells at a significantly lower concentration than 5-FU.

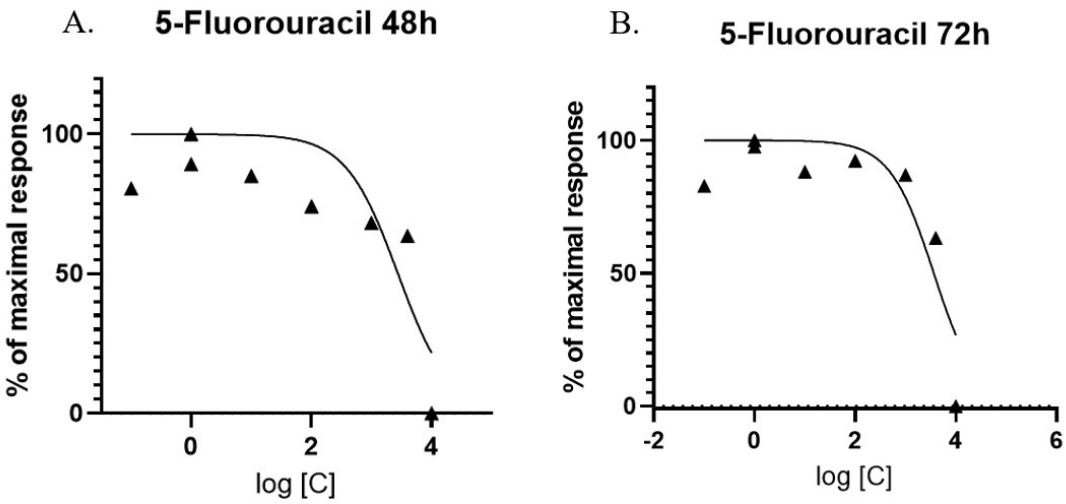

Figure 4. Dose-response curve of of 5-fluorouracil at A. 48h and B. 72h endpoints. Dose-depent cell viability of 4t1-*Luc2* breast tumour cells when treated with different concentrations of 5-fluorouracil was evaluated by Alamar blue viability assay measuring fluoresence at 560 nm at two different endpoints.

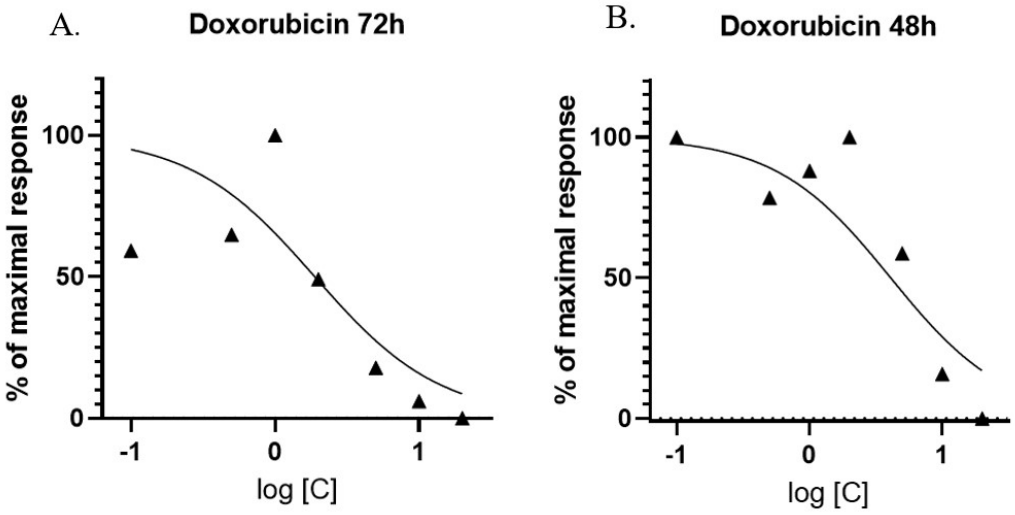

Figure 5. Dose-response curve of of doxorubicin at A. 48h and B. 72h endpoints. Dose-depent cell viability of 4T1-*Luc2* cells when treated with different concentrations of doxorubicin was evaluated by Alamar blue viability assay measuring fluoresence at 560 nm at two different endpoints.

2.3. Altered sensitivity of breast cancer cells to cytotoxic drugs in the artificial bone microenvironment

The altered sensitivity of 4T1-*Luc2* breast cancer cells in artificial bone microenvironment formed by MC3T3-E1 osteoblasts and S53P4 bioactive glass was evaluated by comparing the viability of the cells grown in the artificial bone microenvironment co-culture to the results of the IC50 studies that were cultured solely on plastic (Figures 4 and 5). The results obtained from the luciferase of the lysed 4T1-*Luc2* cells are presented in Figure 6 and Figure 7. As shown in the luciferase assay results, the treatment with 5-FU and doxorubicin reduces the luminescence and thus the numbers of breast cancer cells in the artificial bone microenvironment cell cultures. However, what is most interesting about Figure 6 is the prominent cell death at 10 µM concentration of 5-FU when cultured in the artificial bone microenvironment. When this is compared to the cell death presented in Figure 4 in which the IC50 value of 5-FU was 3,7 mM (4T1-*Luc2* cells cultured solely on plastic). In the IC50 assay, the cytotoxic effect of 5-FU was observed only with the highest concentrations that were tested (Figure 4), while remarkable cell death of breast cancer cells was visible already in a significantly smaller dose of 10 µM when cultured in the artificial bone environment co-culture at the 72h endpoint (Figure 6).

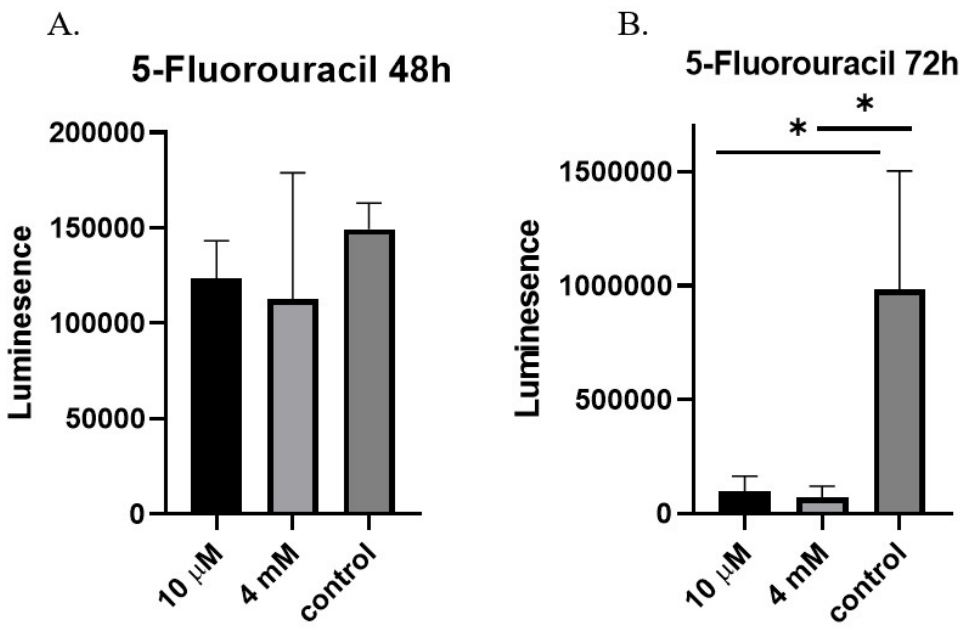

Figure 6. Response of 4T1-*Luc2* cells to 5-fluorouracil in an artificial bone microenvironment measured as luminescence of luciferase activity assay. 4T1-*Luc2* cells were cultured for eight days in an artificial bone microenvironment formed by osteoblasts and S53P4 bioactive glass. Cells were then treated with 5-fluorouracil for 48h (A) or 72h (B) and luciferase reporter assay was conducted from cell lysates. Luminescence was read with VictorX multiplate reader. Untreated cells were used as control. Mean values presented in plot, error bars are for standard deviation and * standing for statistical significance of $p < 0.05$.

This finding of lowered cytotoxic effect compared to the IC50 values was however limited only to 5-FU and no such difference of cytotoxic potential relative to the cell culturing condition was observed with doxorubicin (Figure 7). With doxorubicin, a comparable cytotoxic effect can be seen in both drug concentrations in the 48h endpoint and the 72h endpoint in relation to the determined IC50 values in Table 1 and Figure 7.

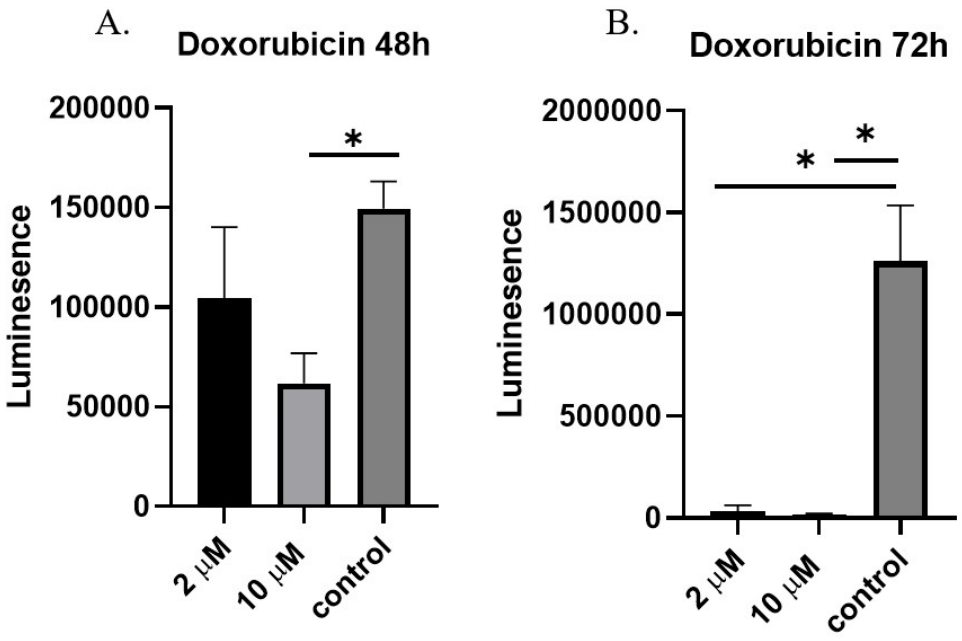

Figure 7. Response of 4T1-*luc2* cells to doxorubicin in an artificial bone microenvironment measured as luminescence of luciferase activity assay. 4T1-*Luc2* cells were cultured for eight days in an artificial bone microenvironment formed by osteoblasts and S53P4 bioactive glass. Cells were then treated with doxorubicin for 48h (A) or 72h (B) and luciferase reporter assay was conducted from cell lysates. Luminescence was read with VictorX multiplate reader. Untreated cells were used as control. Mean values presented in plot, error bars are for standard deviation and * standing for statistical significance of $p < 0.05$.

2.4. Imaging breast tumour colonies in the artificial bone microenvironment

Fluorescent imaging of the fixed and Hoechst-stained cell cultures show tumour colonies formed by the 4T1-*Luc2* breast cancer cells in the artificial bone microenvironment. Figure 8 reveals the breast cancer cells as the dense blue nuclear staining compared to the more elongated and less frequent stained osteoblasts. Bioactive glass granules can be determined by their distinctive borders and sizes. Overall, these images show robust breast cancer cell colonies formed in the artificial bone microenvironment.

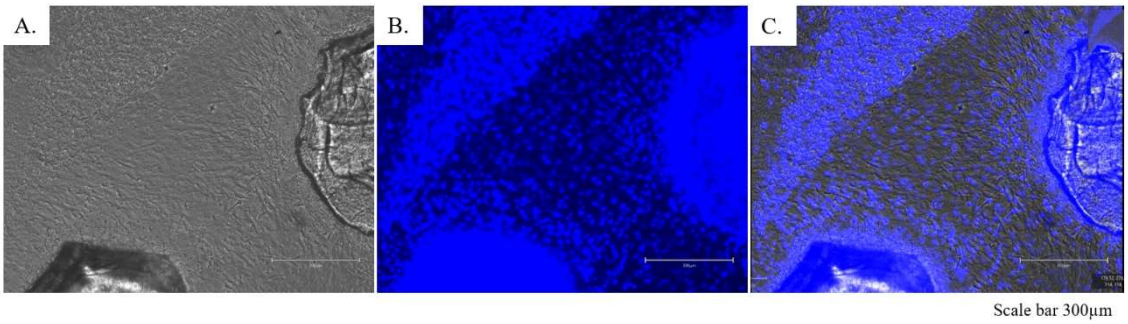

Figure 8. Hoechst-stained cell cultures show breast cancer cell colonies in artificial bone microenvironment co-culture. Fluorescent imaging of Hoechst-stained co-cultures of osteoblasts, S53P4 bioactive glass and 4t1-*luc2* breast cancer cells imaged with EVOS 5000 (10X) at 18th day of culturing, at the end of experiment. A. Brightfield B. Hoechst (DNA stain) C. Overlay

3. Discussion

3.1. S53P4 bioactive glass proved to be a suitable component of *in vitro* bone microenvironment

The results in this thesis project confirmed that the bioactive glass S53P4 can be used together with MC3T3-E1 preosteoblastic cells to model an artificial *in vitro* bone microenvironment in cell culture conditions. As expected, the S53P4 bioactive glass did not disturb the preosteoblast differentiation capabilities. The unidentified matrix deposition near the surface of bioactive glass granules in Figure 3 may indicate formation of the silica layer on the bioactive glass. This finding is consistent with others, who have shown structures formed by the networks of cells producing extracellular matrix on the surface of another type of bioactive glass, Bioglass® 45S5 (Xynos, I. D. et al., 2000). This has not yet been demonstrated in similar detail for the S53P4 bioactive glass but these findings, while preliminary, suggests comparable effects on osteoblasts cultured with the S53P4 granules.

The research design in this thesis study however poses certain uncertainties and themes to develop further. For one, the controls for the bioactive glass should be more carefully chosen in the proceeding studies. Possible controls could include the inert control of aluminium oxide (Al_2O_3). Aluminum oxide is found to be chemically stable, bio-inert and biocompatible material that is used in scaffolds, implants and implantable medical devices and could have been used as an inert control for S53P4 bioactive glass granules. However, even aluminium oxide has been has shown to stimulate differentiation and proliferation of osteoblasts in low concentrations, which might be due to very small Al^{3+} ion leakage into media. (Lau et al., 1991; Karlsson et al., 2003.) This indicates that inert substitutes to be used as controls in cell cultures are extremely difficult to obtain.

3.2. Doxorubicin has a more potent effect on 4T1-*Luc2* cells than 5-FU

The IC50 determination of doxorubicin and 5-FU was conducted as a part of this thesis project to evaluate the effects of the agents on 4T1-*Luc2* cells in normal culturing conditions. Differences between the two drugs activities were noticeable. Examining the IC50 values suggests that doxorubicin needs more time for the toxic effects, since the IC50 value is smaller at the 72h endpoint compared to the 48h endpoint (Table 1), indicating a more potent effect after longer treatment period. In contrast, IC50 values of

5-FU increase with time which could be interpreted as increasing resistance of 4T1-*Luc2* cells to the drug.

It is worth noting that the IC50 curves obtained from in these studies do not represent the optimal dose-response curves and are non-sigmoidal. Similarly, the concentrations of 5-FU needed to exhibit cytotoxic effect in 4T1-*Luc2* cells were unusually high. IC50 values for 5-FU in different cell lines have been reported to be in the range from 0,151 µM to 2,18 mM and doxorubicin to range from 0.00529 µM to 67 µM according to the Genomics of Drug Sensitivity in Cancer Project by the Cancer Genome Project and the Sanger Institute (Cancer Genome Project at the Wellcome Sanger Institute, (UK) and Center for Molecular Therapeutics, Massachusetts General Hospital Cancer Center (USA)., 2020). Instead of interpreting on the IC50 value results as the 4T1-*Luc2* cells being extremely resistant to 5-FU, we must consider that the detected IC50 values propose abnormal circumstances within the studies that included 5-FU. Since the drug concentrations of 5-FU to obtain a cytotoxic effect were unusually high in both IC50 assay and in the artificial bone microenvironment cell culture, it does not seem reasonable that the cell culture environment would cause issues in the drug delivery to the cells that would be demonstrated as a need for high drug concentration. A possible explanation could be that the drug solubility of 5-FU was poor, and the drug was difficult to solubilise that caused the need for uncharacteristically high drug concentrations to establish an effect. Thus, a greater drug panel, wider concentration range and repetitive studies would need to be conducted to provide more reliable results.

3.3. Artificial bone microenvironment may alter sensitivity of 4T1-*Luc2* cells to 5-fluorouracil

Other researchers have also reported that doxorubicin has a higher cytotoxic property than 5-FU and is more effective in reducing multiorgan metastasis induced with 4T1 breast cancer cells *in vivo* (Bao et al., 2011). This effect can be seen in this thesis study as well, when comparing the overall cell death between the two drug groups, where doxorubicin performs better (Figures 6 and 7). What is surprising is the different results in the luciferase reporter assays, which were used to investigate drug sensitivity when treated with either 5-FU or doxorubicin. Breast tumour cells cultured with 5-FU showed increased sensitivity in artificial bone microenvironment when compared to the cells being cultured solely on plastic in the IC50 study. Similar effects were not observed with doxorubicin. This may be explained by differences between doxorubicin's and 5-FU's

different mechanisms of actions. When evaluating the cytotoxic effect of doxorubicin in the 48h co-culture, the concentration in order to induce cytotoxic effect in artificial bone microenvironment needed to be higher (10 µM versus IC50 4,1 µM), indicating an increased drug resistance As stated earlier, 5-FU acts in several ways to disrupt RNA processing and is thought to be effective mainly in the S-phase of cell cycle (Shah and Schwartz, 2001). However, a study using human breast cancer cell line MCF-7 suggests that the cytotoxic effects of 5-FU are present in all the phases of cell cycle instead of being restricted to S-phase (D W Kufe and P P Major, 1981). It can therefore be assumed that the actively proliferative cancer cells in the artificial bone microenvironment are affected more potently by the 5-FU by being exposed to it in all cell cycle phases than by doxorubicin that is effective in only G1-phase.

It should be noted that using untreated cell culture was not the best choice for a control, but the same cytotoxic treatments should have been tested on 4T1-*Luc2* cells that had not been culture in the artificial bone microenvironment. Due to the lack of appropriate controls, we used the IC50 values of 4T1-*Luc2* cells, which were indeed cultured solely on plastic, even though this solution was not ideal regarding the study design.

3.4. Observations of the *in vitro* artificial bone microenvironment study procedures

At the end of experiment of the artificial bone microenvironment, we made a curious observation, as the detachment of cells proved to be problematic. The artificial bone microenvironment cell culture that included osteoblasts, bioactive glass and breast cancer cells was trypsinised at the end of experiment and lysed for the luciferase experiments. Cell detachment using 0.25% Trypsin-EDTA took significantly longer than standard trypsinisation (incubation time approximately 10 min). Also, cells did not detach as individual cells (no suspension), but instead they detached as a whole surface. Similar effect was found by Hanna et al. (2008), with osteogenic differentiation of human adipose mesenchymal stem cells who described it as a “rolled-up carpet”. Hanna et al. concluded that the effect was caused by the trypsin being able to remove connections only between the bottom layer of cells and the cell culture well-plate but not the connections between different layers or the connections between cells. (Hanna et al., 2018) In order to reach complete detachment of cells after mineralisation had begun, the researchers suggested to first treat the cells with EDTA in order to destroy calcium deposits and then with collagenase I to break the peptide bonds of collagens. (Hanna et al., 2018.) Whether the

unsuccessful detachment of cell layers was significant in our luciferase experiments however remains unclear. Cells were lysed before the luciferase reporter assay and since the luminometer was able to detect a luminescence signal, the cell lysis was most likely successful and since all cells were treated with the same protocol, they are also comparable.

3.5. Conclusions

An initial objective of this thesis study was to establish and evaluate the functionality of preosteoblast cell line MC3T3-E1 cultured in the presence of bioactive glass S5P34. The results of this thesis indicate that the bioactive glass and osteoblasts do indeed form a functional environment to model the bone microenvironment, that includes an ECM component and bone cells. No impairment in the osteoblast differentiation was detected and the osteoblasts were able to produce mineralised matrix. The second aim of this study was to investigate whether culturing 4T1-*Luc2* breast cancer cells in the established artificial bone microenvironment affects the sensitivity of breast cancer cells to the cytotoxic agents 5-FU and doxorubicin. The results showed that the breast cancer cells seemed to be increasingly sensitised only to 5-FU and increased their resistance against doxorubicin. These results highlighted that the altered sensitivity is dependent on the drug.

The results presented in this thesis study still need to be repeated and verified before drawing final, reliable conclusions. There are certain issues to overcome, such as possible difficulties with the drug solubility and technical errors within the experiments that included the cytotoxic agents. Also, the study design needs refinement, especially the choice of controls. Overall, this study strengthens the idea that novel methods of breast cancer metastasis modelling are valuable in drug screening purposes and new approaches can and should be developed. The use of S5P34 bioactive glass in cell culture models is an attractive method in modelling bone microenvironment and future research could explore the effects of hypoxia, a known condition in the bone metastases, in this *in vitro* artificial bone microenvironment model.

4. Methods

4.1. Cell lines and culturing conditions

In this thesis study two different cell lines were used, the murine preosteoblastic cell line MC3T3-E1 Subclone 4 (ATCC® CRL2593™) and the murine breast cancer cell line 4T1-*Luc2* (ATCC® CRL2539LUC2™). MC3T3-E1 Subclone 4 has been isolated from the clonal but phenotypically heterogenous MC3T3-E1 cells. MC3T3-E1 Subclone 4 cell line characteristically exhibit high levels of osteoblast differentiation and produce well mineralised ECM. (Wang et al., 1999.)

4T1-*Luc2* is a cell line of murine mammary carcinoma and is shown to spontaneously produce metastatic tumours *in vivo*. The cell line has been transfected with a lentiviral vector containing the firefly luciferase gene (*luc2*) used that creates a stable expression of luciferase enzyme. Luciferase-labelled cancer cells emit light photons that can be accurately detected in *in vitro* bioluminescence assays and imaging.

Cells were stored in liquid nitrogen prior use in cell cultures. Normal culturing media for MC3T3-E1 preosteoblast cells was α-MEM (Gibco, Thermo Fisher™) supplemented with 10% heat inactivated fetal bovine serum (iFBS, USA), 1x penicillin-streptomycin, 1x glutamine (Gibco, Thermo Fisher™) and 10 mM Hepes (Thermo Fisher™). In order to induce osteogenic differentiation, normal culturing media was supplemented with 10 mM Na-β-glycerophosphate, 70 µg/ml ascorbic acid and 10^{-8} M dexamethasone. Dexamethasone was supplemented only for the first three days of cell culturing to initiate the differentiation. Culturing media of 4T1-*Luc2* breast cancer cells was RPMI medium (Thermo Fisher™).) with 10% heat inactivated fetal bovine serum (iFBS, Brazil), 1x penicillin-streptomycin (Gibco, Thermo Fisher™). and 1x glutamine (Gibco, Thermo Fisher™). Cells were cultured in standard conditions of 37°C and 5% CO_2 and growth medium was changed every 2-3 days.

4.2. SP345 bioactive glass

Bioactive glass granules SP345 (BonAlive® Biomaterials Ltd., Turku, Finland) in the size of 500-800 µM were a kind gift from Prof. Pekka Vallittu, University of Turku. The composition of SP345 as weigh percentages is SiO_2 53 %, Na_2O 23 %, CaO 20 % and P_2O_5 4 %. Bioglass granules were heat sterilised prior their use in cell cultures.

4.3. Establishment of *in vitro* artificial bone microenvironment

The bioactive glass granules were conditioned before introducing MC3T3-E1 preosteoblasts to the culture. This was performed as a precaution to reduce the cellular stress caused by the anticipated increase in the pH due to the release of ions from the bioactive glass. 15 µg of SP345 bioactive glass granules were placed in each well of a 24-well plate (Nunc™, Thermo Fisher™) in growth media for 24h before seeding preosteoblastic cells. MC3T3-E1 preosteoblasts were seeded the following day at a 10 000/cm^2 density (10 000 per well) and growth medium was changed to the differentiating osteogenic culture medium. MC3T3-E1 cells were cultured together with SP345 bioactive glass to form the artificial bone microenvironment and the cell cultures were continued to either evaluate the osteogenic capacity staining studies or to perform co-culture studies with 4T1-*Luc2* cells.

4.4. Osteoblast staining experiments

After eight days of culturing MC3T3-E1 osteoblast cells with bioactive glass S53P4 in differentiating culturing conditions (as described above), ALP staining was performed to obtain qualitative data of osteoblast differentiation. Cells were first washed 3x in Phosphate-buffered saline (PBS) and the fixed in 4% paraformaldehyde (PFA) (200 µl/well) for 15 minutes (min). After fixation, washes with PBS were repeated three times. Leukocyte Alkaline phosphatase kit 86R (Sigma Aldrich) was used for ALP staining according to manufacturer's instructions. FRV-Alkaline solution and sodium-nitrite solution were mixed in a 1:1 ratio of 100 µl and incubated for 2 min at room temperature (RT). The mixture was then added to 4,5 ml of 37°C prewarmed distilled water (dH_2O). 100 µl Naphthol AS-BI Alkaline solution was then added to the mixture and mixed, and the total volume of final solution was 4,8 ml.

200 µl per well of final solution was added to fixed cells and incubated for 15 min at RT in the dark. After incubation, the reaction was stopped by removing the solution and washing cells with dH_2O and air dried.

von Kossa staining was used to verify mineralisation capabilities of MC3T3-E1 osteoblast cells in the cell culture after 21 days of culturing cells with bioactive glass S53P4 in osteogenic differentiation conditions. von Kossa staining is used to detect calcium deposits and is an indication of successful *in vitro* bone formation. Cells were first washed 3x in PBS and the cell cultures were fixed in 4% PFA (200 µl/well) for 15

min. Cells were then washed 3x with PBS and consequently washed 2x with dH_2O. Cells were next treated with 200 µl per well of 2% silver nitrate ($AgNO_3$) solution and incubated for 60 min while exposed to a 60W lamp. Cells were consequently washed 3x with dH_2O and 200 µl of 2.5% sodium thiosulfate solution was added on cells per well and incubated for 5 min at RT. Cells were subsequently washed with dH_2O and air dried.

4.5. IC50 assays of 5-FU and doxorubicin

The pharmaceutical agents that were used in the IC50 experiments were 5-FU (Sigma Aldrich, F6627, lot MKBX3795V) in DMSO and doxorubicin, a kind gift form prof. Johanna Tuomela, University of Turku. IC50 values of 4T1-*Luc2* cells for doxorubicin and 5-FU were determined with an Alamar blue cell viability assay. Assay was done twice with two different sets of drug concentrations due to extremely low cytotoxic effect of the drugs in the first round of experiments (results are not shown).

4T1-*Luc2* cells lines were plated on 96-well plate (Nunc™, Thermo Fisher™) at a seeding density of 1000 cells/well in 100 µl of RPMI growth medium and cells were then incubated overnight at 37°C in 5% CO_2. Cells were treated with either doxorubicin or 5-FU with concentration series described in Table 2, in quadruples for 48h or for 72h. After treatment period, 10 µl of AlamarBlue cell viability reagent (Thermo Fischer) was added to each well, including blanks, and incubated for 2h at 37°C in 5% CO_2. Fluorescence was subsequently read at 560 nm using HIDEX Plate Chameleon^{TM} Reader (Hidex Ltd., Turku, Finland).

Table 2. Drug concentrations of doxorubicin and 5-fluorouracil for the IC50 experiments on 4T1-*Luc2* cells

Doxorubicin (c)	0.1 µM	0.5 µM	2 µM	5 µM	10 µM	20 µM	
5-fluorouracil (c)	0.1 µM	1 µM	10 µM	100 µM	1 mM	2 mM	10 mM

4.6. Artificial bone microenvironment co-culture with 4T1-*Luc2* cells

Artificial bone microenvironment was established of by culturing MC3T3-E1 osteoblast cells with bioactive glass S53P4 in the osteoblast differentiating culturing conditions as described in Chapter 4.3. After eight days of culturing S53P4 bioactive glass and MC3T3-E1 osteoblasts, 500 4T1-*Luc2* cells were plated into artificial bone microenvironment as triplicates and the co-culture was let grow in the osteogenic media for additional eight

days. Medium was changed every 2-3 days. On day 16, the co-cultures were treated with 2 µM or 10 µM doxorubicin or with 10 µM or 4mM 5-FU for 48h or 72h. After 48h or 72h, cell cultures were detached with 0.25% trypsin-EDTA solution and the cells were lysed with Tropix lysis solution (Applied Biosystems, Life Technologies). Hoechst-staining (see below) was additionally performed. Overview of the co-culturing flow of S53P4 bioactive glass, MC3T3-E1 osteoblasts and 4T1-*Luc2* breast cancer cells is presented in Figure 9.

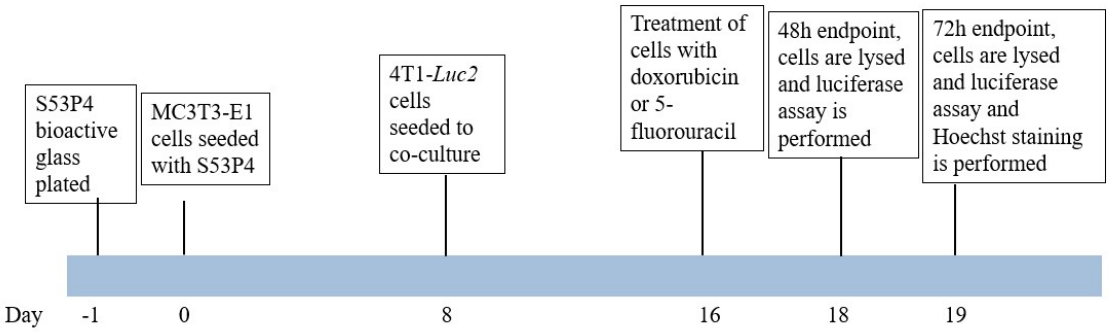

Figure 9. Overview of study outline of 4T1-*Luc2* breast cancer cells in artificial bone microenvironment co-culture with S53P4 bioactive glass and MC3T3-E1 osteoblasts.

Hoechst staining of the co-culture was conducted at the 72h endpoint. Cells were first washed 3x in PBS and then fixed in 4% PFA for 15 min. Cells were washed 3x with PBS and then stained with Hoechst 33258 (Sigma Aldrich), a blue fluorescent stain specific for DNA marking the nucleus of the cells. 5 µM of Hoechst dye was added to each well and incubated for 15 min, RT. Cells were then washed 3x with PBS and fluorescent imaging was conducted with EVOS M5000 Imaging system (Invitrogen™).

4.7. Luciferase reporter assay of 4T1-*Luc2* cells

Luciferase assay with the 4T1-*Luc2* cells was first performed first without administration of any pharmaceutical agents and with calculated cell numbers to verify that measuring luminescence was a suitable method to assess cell numbers in culture. 4T1-*Luc2* cells were grown in the RPMI growth medium up to confluency and detached with 0.25% trypsin-EDTA solution. The solution was inactivated with growth medium and cells were calculated using Bürker-Türk cell calculation chamber. 10 000 cells per 10 µl was then lysed with Tropix lysis solution and cell lysis was added to 96-well microplates as two-fold serial dilution to also get 5000 and 2500 cells per well. For the luciferase assay, we used the Luciferase Assay Kit No. 484-001 by BioThema. 100 µl of luciferin substrate

and 100 µl of ATP substrate was consequently added to each well and luminescence was measured with VictorX multiplate reader (PerkinElmer). These results are included in Supplementary Figure 1.

At end of experiment the co-cultured 4T1-*Luc2* cells were lysed with Tropix lysis solution. For the luciferase assay we used the Luciferase Assay Kit No. 484-001 by BioThema.10 µl of lysed sample was added to each well of a 96-well microplate as triplicates. 100 µl of luciferin substrate and 100 µl of ATP substrate was consequently added to each well and luminescence was measured with VictorX multiplate reader.

4.8. Statistical analysis

All data was analysed with GraphPad Prism version 8.4 for Windows. The IC50 assay results were determined by transforming values to a logarithmic scale and fitting the values into a nonlinear regression curve. Luciferase activity assay was analysed using One-way Anova and comparisons between different treatment concentrations and controls were analysed using Tukey´s multiple comparisons test. Staining images were handled using QuPath. version 0.1.2.

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6. Abbreviations

5-FU	5-fluorouracil
ALP	Alkaline phosphatase
BMP	Bone morphogenetic protein
CTC	Circulating tumour cell
CXCL12	Chemokine ligand 12
DTC	Disseminating tumour cell
EMT	Epithelial-Mesenchymal transition
IGF	Insulin-like growth-factor
PTHrP	Parathyroid hormone-related protein
RANK	Receptor activator of nuclear factor κB
RANKL	Receptor activator of nuclear factor κB ligand
Runx2	Runt-related transcription factor 2
RT	Room temperature
SRE	Skeletal related event
TS	Thymidylate synthase

7. References

Aguirre-Ghiso, J.A. 2007. Models, mechanisms and clinical evidence for cancer dormancy. *Nature Reviews. Cancer.* 7:834-846. doi: 10.1038/nrc2256.

Alba, E., M. Martín, M. Ramos, E. Adrover, A. Balil, C. Jara, A. Barnadas, A. Fernández-Aramburo, P. Sánchez-Rovira, M. Amenedo, and A. Casado. 2004. Multicenter Randomized Trial Comparing Sequential With Concomitant Administration of Doxorubicin and Docetaxel As First-Line Treatment of Metastatic Breast Cancer: A Spanish Breast Cancer Research Group (GEICAM-9903) Phase III Study. *Jco.* 22:2587-2593. doi: 10.1200/JCO.2004.08.125.

Alison T. Stopeck, Allan Lipton, Jean-Jacques Body, Guenther G. Steger, Katia Tonkin, Richard H. de Boer, Mikhail Lichinitser, Yasuhiro Fujiwara, Denise A. Yardley, María Viniegra, Michelle Fan, Qi Jiang, Roger Dansey, Susie Jun, and Ada Braun. 2010. Denosumab Compared With Zoledronic Acid for the Treatment of Bone Metastases in Patients With Advanced Breast Cancer: A Randomized, Double-Blind Study. *Journal of Clinical Oncology.* 28:5132-5139. doi: 10.1200/JCO.2010.29.7101.

American Cancer Society. 2018. Global Cancer Facts & Figures 4th Edition. *American Cancer Society.*

Attardi, L.D., A. de Vries, and T. Jacks. 2004. Activation of the p53-dependent G1 checkpoint response in mouse embryo fibroblasts depends on the specific DNA damage inducer. *Oncogene.* 23:973-980. doi: 10.1038/sj.onc.1207026.

Aubel-Sadron, G., and D. Londos-Gagliardi. 1984. Daunorubicin and doxorubicin, anthracycline antibiotics, a physicochemical and biological review. *Biochimie.* 66:333-352. doi: 10.1016/0300-9084(84)90018-x.

Bao, L., A. Haque, K. Jackson, S. Hazari, K. Moroz, R. Jetly, and S. Dash. 2011. Increased Expression of P-Glycoprotein Is Associated with Doxorubicin Chemoresistance in the Metastatic 4T1 Breast Cancer Model. *American Journal of Pathology, The.* 178:838-852. doi: 10.1016/j.ajpath.2010.10.029.

Bhadriraju, K., and C.S. Chen. 2002. Engineering cellular microenvironments to improve cell-based drug testing. *Drug Discovery Today.* 7:612-620.

Boyce, B.F., and L. Xing. 2008. Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Archives of Biochemistry and Biophysics.* 473:139. doi: 10.1016/j.abb.2008.03.018.

Braun, S., F.D. Vogl, B. Naume, W. Janni, M.P. Osborne, R.C. Coombes, G. Schlimok, I.J. Diel, B. Gerber, G. Gebauer, J. Pierga, C. Marth, D. Oruzio, G. Wiedswang, E. Solomayer, G. Kundt, B. Strobl, T. Fehm, G.Y.C. Wong, J. Bliss, A. Vincent-Salomon, and K. Pantel. 2005. A Pooled Analysis of Bone Marrow Micrometastasis in Breast Cancer. *The New England Journal of Medicine.* 353:793-802. doi: 10.1056/NEJMoa050434.

Breslin, S., and L. O'Driscoll. 2013. Three-dimensional cell culture: the missing link in drug discovery. *Drug Discovery Today.* 18:240-249. doi: 10.1016/j.drudis.2012.10.003.

Bussard, K., K. Bussard, C. Gay, C. Gay, A. Mastro, and A. Mastro. 2008. The bone microenvironment in metastasis; what is special about bone? *Cancer Metastasis Rev.* 27:41-55. doi: 10.1007/s10555-007-9109-4.

Cameron, D.A., H. Gabra, and R.C. Leonard. 1994. Continuous 5-fluorouracil in the treatment of breast cancer. *British Journal of Cancer.* 70:120-124. doi: 10.1038/bjc.1994.259.

Cancer Genome Project at the Wellcome Sanger Institute, (UK), and Center for Molecular Therapeutics, Massachusetts General Hospital Cancer Center (USA). 2020. Genomics of Drug Sensitivity in Cancer. 2020.

Christensen, M.H., and L.J. Petersen. 2011. Radionuclide treatment of painful bone metastases in patients with breast cancer: A systematic review. *Cancer Treatment Reviews.* 38:164-171. doi: 10.1016/j.ctrv.2011.05.008.

D W Kufe, and P P Major. 1981. 5-Fluorouracil incorporation into human breast carcinoma RNA correlates with cytotoxicity. *Journal of Biological Chemistry.* 256:9802.

Diel, I.J., E. Solomayer, S.D. Costa, C. Gollan, R. Goerner, D. Wallwiener, M. Kaufmann, and G. Bastert. 1998. Reduction in New Metastases in Breast Cancer with Adjuvant Clodronate Treatment. *The New England Journal of Medicine.* 339:357-363. doi: 10.1056/NEJM199808063390601.

Dunn, L.K., K.S. Mohammad, P.G.J. Fournier, C.R. McKenna, H.W. Davis, M. Niewolna, X.H. Peng, J.M. Chirgwin, and T.A. Guise. 2009. Hypoxia and TGF-β Drive Breast Cancer Bone Metastases through Parallel Signaling Pathways in Tumor Cells and the Bone Microenvironment. *Plos One.* 4:e6896. doi: 10.1371/journal.pone.0006896.

Fournier, P.G.J., P. Juárez, and T.A. Guise. 2015. Tumor–bone interactions: there is no place like bone. *In* Bone Cancer. . 13-28.

Gaze, M.N., C.G. Kelly, G.R. Kerr, A. Cull, V.J. Cowie, A. Gregor, G.C.W. Howard, and A. Rodger. 1997. Pain relief and quality of life following radiotherapy for bone metastases: a randomised trial of two fractionation schedules. *Radiotherapy and Oncology.* 45:109-116. doi: 10.1016/S0167-8140(97)00101-1.

Giorgio Minotti, Pierantonio Menna, Emanuela Salvatorelli, Gaetano Cairo, and Luca Gianni. 2004. Anthracyclines: Molecular Advances and Pharmacologic Developments in Antitumor Activity and Cardiotoxicity. *Pharmacological Reviews.* 56:185-229. doi: 10.1124/pr.56.2.6.

Greenberg, P.A., G.N. Hortobagyi, T.L. Smith, L.D. Ziegler, D.K. Frye, and A.U. Buzdar. 1996. Long-term follow-up of patients with complete remission following

combination chemotherapy for metastatic breast cancer. *Jco.* 14:2197-2205. doi: 10.1200/JCO.1996.14.8.2197.

Hanahan, D., and R. Weinberg. 2011. Hallmarks of Cancer: The Next Generation. *Cell (Cambridge).* 144:646-674. doi: 10.1016/j.cell.2011.02.013.

Hanna, H., L.M. Mir, and F.M. Andre. 2018. In vitro osteoblastic differentiation of mesenchymal stem cells generates cell layers with distinct properties. *Stem Cell Research & Therapy.* 9:203. doi: 10.1186/s13287-018-0942-x.

Harada, S., and G.A. Rodan. 2003. Control of osteoblast function and regulation of bone mass. *Nature (London).* 423:349-355. doi: 10.1038/nature01660.

Harbeck, N., and M. Gnant. 2017. Breast cancer. *The Lancet.* 389:1134-1150. doi: 10.1016/S0140-6736(16)31891-8.

Harries, M., A. Taylor, L. Holmberg, O. Agbaje, H. Garmo, S. Kabilan, and A. Purushotham. 2014. Incidence of bone metastases and survival after a diagnosis of bone metastases in breast cancer patients. *Cancer Epidemiology.* 38:427-434. doi: 10.1016/j.canep.2014.05.005.

Heidelberger, C., N.K. CHAUDHURI, P. DANNEBERG, D. MOOREN, L. GRIESBACH, R. DUSCHINSKY, R.J. SCHNITZER, E. PLEVEN, and J. SCHEINER. 1957. Fluorinated Pyrimidines, A New Class of Tumour-Inhibitory Compounds. *Nature (London).* 179:663-666. doi: 10.1038/179663a0.

Heino, T., and J. Määttä. 2018. Bone Marrow Niche: Role of Different Cells in Bone Metastasis. *Curr Mol Bio Rep.* 4:80-87. doi: 10.1007/s40610-018-0091-0.

Hiraga, T. 2019. Bone metastasis: Interaction between cancer cells and bone microenvironment. *Journal of Oral Biosciences.* 61:95-98. doi: 10.1016/j.job.2019.02.002.

Hiraga, T. 2018. Hypoxic Microenvironment and Metastatic Bone Disease. *International Journal of Molecular Sciences.* 19. doi: 10.3390/ijms19113523.

Hiraga, T., A. Myoui, N. Hashimoto, A. Sasaki, K. Hata, Y. Morita, H. Yoshikawa, C.J. Rosen, G.R. Mundy, and T. Yoneda. 2012. Bone-derived IGF mediates crosstalk between bone and breast cancer cells in bony metastases. *Cancer Research.* 72:4238. doi: 10.1158/0008-5472.CAN-11-3061.

Holen, I., F. Nutter, J. Wilkinson, C. Evans, P. Avgoustou, and P. Ottewell. 2015. Human breast cancer bone metastasis in vitro and in vivo: a novel 3D model system for studies of tumour cell-bone cell interactions. *Clin Exp Metastasis.* 32:689-702. doi: 10.1007/s10585-015-9737-y.

Holle, A.W., J.L. Young, and J.P. Spatz. 2016. In vitro cancer cell–ECM interactions inform in vivo cancer treatment. *Advanced Drug Delivery Reviews.* 97:270-279. doi: 10.1016/j.addr.2015.10.007.

Hortobagyi, G.N., R.L. Theriault, L. Porter, D. Blayney, A. Lipton, C. Sinoff, H. Wheeler, J.F. Simeone, J. Seaman, R.D. Knight, M. Heffernan, D.J. Reitsma, I. Kennedy, S.G. Allan, and K. Mellars. 1996. Efficacy of Pamidronate in Reducing Skeletal Complications in Patients with Breast Cancer and Lytic Bone Metastases. *The New England Journal of Medicine.* 335:1785-1792. doi: 10.1056/NEJM199612123352401.

Ina Kramer, Christine Halleux, Hansjoerg Keller, Marco Pegurri, Jonathan H. Gooi, Patricia Brander Weber, Jian Q. Feng, Lynda F. Bonewald, and Michaela Kneissel. 2010. Osteocyte Wnt/β-Catenin Signaling Is Required for Normal Bone Homeostasis. *Molecular and Cellular Biology.* 30:3071-3085. doi: 10.1128/MCB.01428-09.

Jinnah, A.H., B.C. Zacks, C.U. Gwam, and B.A. Kerr. 2018. Emerging and Established Models of Bone Metastasis. *Cancers.* 10:176. doi: 10.3390/cancers10060176.

Kang, Y., P.M. Siegel, W. Shu, M. Drobnjak, S.M. Kakonen, C. Cordón-Cardo, T.A. Guise, and J. Massagué. 2003. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell.* 3:537-549. doi: 10.1016/s1535-6108(03)00132-6.

Karlsson, M., E. Pålsgård, P.R. Wilshaw, and L. Di Silvio. 2003. Initial in vitro interaction of osteoblasts with nano-porous alumina. *Biomaterials.* 24:3039-3046. doi: 10.1016/S0142-9612(03)00146-7.

Kenny, P.A., G.Y. Lee, C.A. Myers, R.M. Neve, J.R. Semeiks, P.T. Spellman, K. Lorenz, E.H. Lee, M.H. Barcellos-Hoff, O.W. Petersen, J.W. Gray, and M.J. Bissell. 2007. The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Molecular Oncology.* 1:84-96. doi: 10.1016/j.molonc.2007.02.004.

Kodama, H., M. Nose, S. Niida, and A. Yamasaki. 1991. Essential role of macrophage colony-stimulating factor in the osteoclast differentiation supported by stromal cells. *The Journal of Experimental Medicine.* 173:1291-1294. doi: 10.1084/jem.173.5.1291.

Komori, T., H. Yagi, S. Nomura, A. Yamaguchi, K. Sasaki, K. Deguchi, Y. Shimizu, R.T. Bronson, Y.-. Gao, M. Inada, M. Sato, R. Okamoto, Y. Kitamura, S. Yoshiki, and T. Kishimoto. 1997. Targeted Disruption of Cbfa1 Results in a Complete Lack of Bone Formation owing to Maturational Arrest of Osteoblasts. *Cell (Cambridge).* 89:755-764. doi: 10.1016/s0092-8674(00)80258-5.

Kuchuk, I., B. Hutton, P. Moretto, T. Ng, C.L. Addison, and M. Clemons. 2013. Incidence, consequences and treatment of bone metastases in breast cancer patients—Experience from a single cancer centre. *Journal of Bone Oncology.* 2:137-144. doi: 10.1016/j.jbo.2013.09.001.

Kwakwa, K.A., and J.A. Sterling. 2017. Integrin αvβ3 Signaling in Tumor-Induced Bone Disease. *Cancers (Basel).* 9. doi: 10.3390/cancers9070084.

Lau, K.H., A. Yoo, and S.P. Wang. 1991. Aluminum stimulates the proliferation and differentiation of osteoblasts in vitro by a mechanism that is different from fluoride. *Molecular and Cellular Biochemistry.* 105:93. doi: 10.1007/BF00227749.

Liang, Y., H. Zhang, X. Song, and Q. Yang. 2020. Metastatic heterogeneity of breast cancer: Molecular mechanism and potential therapeutic targets. *Seminars in Cancer Biology.* 60:14-27. doi: 10.1016/j.semcancer.2019.08.012.

Lindfors, N.C., I. Koski, J.T. Heikkilä, K. Mattila, and A.J. Aho. 2010. A prospective randomized 14-year follow-up study of bioactive glass and autogenous bone as bone graft substitutes in benign bone tumors. *Journal of Biomedical Materials Research Part B: Applied Biomaterials.* 94B:157-164. doi: 10.1002/jbm.b.31636.

Long, F. 2011. Building strong bones: molecular regulation of the osteoblast lineage. *Nature Reviews. Molecular Cell Biology.* 13:27-38. doi: 10.1038/nrm3254.

Longley, D.B., D.P. Harkin, and P.G. Johnston. 2003. 5-Fluorouracil: mechanisms of action and clinical strategies. *Nature Reviews Cancer.* 3:330-338. doi: 10.1038/nrc1074.

Müller, A., B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S.N. Wagner, J.L. Barrera, A. Mohar, E. Verástegui, and A. Zlotnik. 2001. Involvement of chemokine receptors in breast cancer metastasis. *Nature (London).* 410:50-56. doi: 10.1038/35065016.

Mundy, G.R. 2002. Metastasis to bone: causes, consequences and therapeutic opportunities. *Nature Reviews. Cancer.* 2:584-593. doi: 10.1038/nrc867.

Nakashima, K., X. Zhou, G. Kunkel, Z. Zhang, J.M. Deng, R.R. Behringer, and B. de Crombrugghe. 2002. The Novel Zinc Finger-Containing Transcription Factor Osterix Is Required for Osteoblast Differentiation and Bone Formation. *Cell.* 108:17-29. doi: 10.1016/S0092-8674(01)00622-5.

O'Shaughnessy, J. 2005. Extending Survival with Chemotherapy in Metastatic Breast Cancer. *The Oncologist (Dayton, Ohio).* 10:20-29. doi: 10.1634/theoncologist.10-90003-20.

Page, J.M., A.R. Merkel, N.S. Ruppender, R. Guo, U.C. Dadwal, S. Cannonier, S. Basu, S.A. Guelcher, and J.A. Sterling. 2015. Matrix Rigidity Regulates the Transition of Tumor Cells to a Bone-Destructive Phenotype through Integrin β3 and TGF-β Receptor Type II. *Biomaterials.* 64:33-44. doi: 10.1016/j.biomaterials.2015.06.026.

Pecheur, I., O. Peyruchaud, C. Serre, J. Guglielmi, C. Voland, F. Bourre, C. Margue, M. Cohen-Solal, A. Buffet, N. Kieffer, and P. CléZardin. 2002. Integrin αvβ3 expression confers on tumor cells a greater propensity to metastasize to bone. *The FASEB Journal.* 16:1266-1268. doi: 10.1096/fj.01-0911fje.

Peltola, M., K. Aitasalo, J. Suonpää, M. Varpula, and A. Yli-Urpo. 2006. Bioactive glass S53P4 in frontal sinus obliteration: A long-term clinical experience. *Head & Neck.* 28:834-841. doi: 10.1002/hed.20436.

Popovic, M., R. Chow, N. Lao, G. Bedard, and E. Chow. 2015. Pain control with palliative radiotherapy in patients with bone metastases. *In* Bone Cancer. . 599-613.

Powell, G.J., J. Southby, J.A. Danks, R.G. Stillwell, J.A. Hayman, M.A. Henderson, R.C. Bennett, and T.J. Martin. 1991. Localization of Parathyroid Hormone-related Protein in Breast Cancer Metastases: Increased Incidence in Bone Compared with Other Sites. *Cancer Research.* 51:3059-3061.

Qiao, H., and T. Tang. 2018. Engineering 3D approaches to model the dynamic microenvironments of cancer bone metastasis. *Bone Research.* 6:3-12. doi: 10.1038/s41413-018-0008-9.

Regazzoni, S., G. Pesce, G. Marini, F. Cavalli, and A. Goldhirsch. 1996. Low-dose continuous intravenous infusion of 5-fluorouracil for metastatic breast cancer. *Annals of Oncology.* 7:807-813. doi: 10.1093/oxfordjournals.annonc.a010759.

Roach, H. 1999. Association of Matrix Acid and Alkaline Phosphatases with Mineralization of Cartilage and Endochondral Bone. *Histochem J.* 31:53-61. doi: 10.1023/A:1003519104980.

Roodman, G.D. 2004. Mechanisms of Bone Metastasis. *The New England Journal of Medicine.* 350:1655-1664. doi: 10.1056/NEJMra030831.

Shah, M.A., and G.K. Schwartz. 2001. Cell cycle-mediated drug resistance : An emerging concept in cancer therapy. *Clinical Cancer Research.* 7:2168-2181.

Sharma, S.V., D.A. Haber, and J. Settleman. 2010. Cell line-based platforms to evaluate the therapeutic efficacy of candidate anticancer agents. *Nature Reviews. Cancer.* 10:241-253. doi: 10.1038/nrc2820.

Slamon, D.J., B. Leyland-Jones, S. Shak, H. Fuchs, V. Paton, A. Bajamonde, T. Fleming, W. Eiermann, J. Wolter, M. Pegram, J. Baselga, and L. Norton. 2001. Use of Chemotherapy plus a Monoclonal Antibody against HER2 for Metastatic Breast Cancer That Overexpresses HER2. *The New England Journal of Medicine.* 344:783-792. doi: 10.1056/NEJM200103153441101.

Swain, S.M., J. Baselga, S. Kim, J. Ro, V. Semiglazov, M. Campone, E. Ciruelos, J. Ferrero, A. Schneeweiss, S. Heeson, E. Clark, G. Ross, M.C. Benyunes, and J. Cortés. 2015. Pertuzumab, Trastuzumab, and Docetaxel in HER2-Positive Metastatic Breast Cancer. *The New England Journal of Medicine.* 372:724-734. doi: 10.1056/NEJMoa1413513.

Tzelepi, V., A.C. Tsamandas, V. Zolota, and C.D. Scopa. 2009. Bone Anatomy, Physiology and Function. *In* Bone Metastases: A Translational and Clinical Approach. D. Kardamakis, V. Vassiliou and E. Chow, editors. Springer Netherlands, Dordrecht. 3-30.

Välimäki, V.-., and H.T. Aro. 2006. Molecular Basis for Action of Bioactive Glasses as Bone Graft Substitute. *Scandinavian Journal of Surgery.* 95:95-102. doi: 10.1177/145749690609500204.

van Gestel, N. A. P., J. Geurts, D.J.W. Hulsen, B. van Rietbergen, S. Hofmann, and J.J. Arts. 2015. Clinical Applications of S53P4 Bioactive Glass in Bone Healing and

Osteomyelitic Treatment: A Literature Review. *Biomed Res Int.* 2015. doi: 10.1155/2015/684826.

van Golen, C.M., T.S. Schwab, B. Kim, M.E. Soules, S. Su Oh, K. Fung, K.L. van Golen, and E.L. Feldman. 2006. Insulin-Like Growth Factor-I Receptor Expression Regulates Neuroblastoma Metastasis to Bone. *Cancer Research.* 66:6570-6578. doi: 10.1158/0008-5472.CAN-05-1448.

Vimalraj, S. 2020. Alkaline phosphatase: Structure, expression and its function in bone mineralization. *Gene.* 754:144855. doi: 10.1016/j.gene.2020.144855.

Waks, A.G., and E.P. Winer. 2019. Breast Cancer Treatment: A Review. *JAMA : The Journal of the American Medical Association.* 321:288-300. doi: 10.1001/jama.2018.19323.

Wang, D., K. Christensen, K. Chawla, G. Xiao, P.H. Krebsbach, and R.T. Franceschi. 1999. Isolation and Characterization of MC3T3-E1 Preosteoblast Subclones with Distinct In Vitro and In Vivo Differentiation/Mineralization Potential. *Journal of Bone and Mineral Research.* 14:893-903. doi: 10.1359/jbmr.1999.14.6.893.

Woelfle, U., J. Cloos, G. Sauter, L. Riethdorf, F. Janicke, P. van Diest, R. Brakenhoff, and K. Pantel. 2003. Molecular Signature Associated with Bone Marrow Micrometastasis in Human Breast Cancer. *Cancer Research.* 63:5679-5684.

Wong, C.H., K.W. Siah, and A.W. Lo. 2019. Estimation of clinical trial success rates and related parameters. *Biostatistics (Oxford, England).* 20:273-286. doi: 10.1093/biostatistics/kxx069.

Xynos, I.D., M.V.J. Hukkanen, J.J. Batten, L.D. Buttery, L.L. Hench, and J.M. Polak. 2000. Bioglass ®45S5 Stimulates Osteoblast Turnover and Enhances Bone Formation In Vitro: Implications and Applications for Bone Tissue Engineering. *Calcif Tissue Int.* 67:321-329. doi: 10.1007/s002230001134.

Xynos, I.D., A.J. Edgar, L.D.K. Buttery, L.L. Hench, and J.M. Polak. 2000. Ionic Products of Bioactive Glass Dissolution Increase Proliferation of Human Osteoblasts and Induce Insulin-like Growth Factor II mRNA Expression and Protein Synthesis. *Biochemical and Biophysical Research Communications.* 276:461-465. doi: 10.1006/bbrc.2000.3503.

Yin, J.J., K. Selander, J.M. Chirgwin, M. Dallas, B.G. Grubbs, R. Wieser, J. Massagué, G.R. Mundy, and T.A. Guise. 1999. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *The Journal of Clinical Investigation.* 103:197-206. doi: 10.1172/JCI3523.

Young, B., G. O'Dowd, and P. Woodford. 2014. Wheater's Functional Histology. Churchill Livingstone. 180 pp.

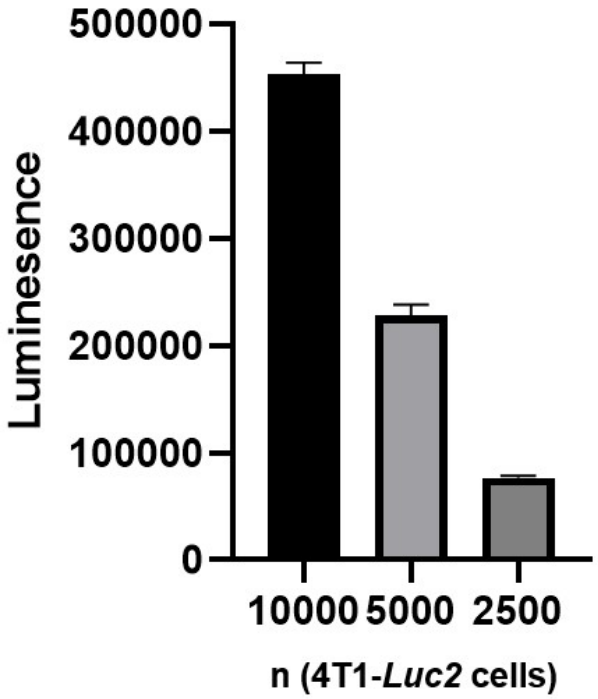
Zhong, H., A.M. De Marzo, E. Laughner, M. Lim, D.A. Hilton, D. Zagzag, P. Buechler, W.B. Isaacs, G.L. Semenza, and J.W. Simons. 1999. Overexpression of Hypoxia-

inducible Factor 1α in Common Human Cancers and Their Metastases. *Cancer Res.* 59:5830-5835.

9. Supplementary Material

9.1. Luciferase assay of 4T1-*Luc2* cells

In order to assess whether the measuring of luminescence from the 4T1-*Luc2* cells was sensitive enough methods to evaluate and compare the number of cells in the experiments, an additional luciferase assay was made. In this experiment, a known number of 4T1-*Luc2* cells were lysed and the luminescence was measured. As shown in Supplementary Figure 1, the number of cells correlates with the measured luminesce values.



Supplementary Figure 1. Number of 4T1-*Luc*2 cells correlate with luminescence. After culturing 4T1-*Luc*2 cells in normal conditions cells were calculated and lysed, 10 000 cells per 10 µl lysis. Two-fold serial dilutions were conducted, and luciferase assay was performed. Luminescence was measured with VictorX multiplate reader.