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Molecular alterations that drive breast cancer metastasis to bone

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Abstract

Epithelial cancers including breast and prostate commonly progress to form incurable bone metastases. For this to occur cancer cells must adapt their phenotype and behaviour to enable detachment from the primary tumour, invasion into the vasculature, homing to and subsequent colonisation of bone. It is widely accepted that the metastatic process is driven by the transformation of cancer cells from a sessile epithelial to a motile mesenchymal phenotype through epithelial-mesenchymal transition (EMT). Dissemination of these motile cells into the circulation provides the conduit for cells to metastasise to distant organs. However, accumulating evidence suggests that EMT is not sufficient for metastasis to occur and specific tissue homing factors are required for tumour cells to lodge and grow in bone. Once tumour cells are disseminated in the bone environment they can revert back into an epithelial phenotype through the reverse process of mesenchymal-epithelial transition (MET) and form secondary tumours. In this review we describe the molecular alterations undertaken by breast cancer cells at each stage of the metastatic cascade and discuss how these changes facilitate bone metastasis.

Introduction

Breast cancer is the most frequently diagnosed cancer and leading cause of cancer death in females worldwide[1]. Despite this, the majority of primary tumours that remain confined to the breast are amenable to currently available treatments and 5-year survival rates for patients with non-metastatic disease is ~93%. However, once the tumour has metastasised to a distant site 5-year survival decreases to ~22% (National Cancer Institute SEER database). For breast cancer the most common site of metastasis is bone and patients with this condition have a median survival of around 2-3 years following initial diagnosis of bone involvement. Identification of new therapeutic approaches are therefore needed to improve outcome for patients

with tumour spread to the skeleton. A better understanding of the molecular determinants that drive the different stages of breast cancer metastasis to bone are essential for the future development of successful therapeutic strategies.

Metastatic conversion of breast cancer cells is driven by genetic and phenotypic adaptations that change tumour cells from an epithelial to a mesenchymal phenotype (EMT). This process is initiated by the overexpression of mesenchymal proteins such as fibronectin and metalloproteinases[2-3] in addition to the loss of cell adhesion molecules including E-cadherin and B-cadherin. Loss of E-cadherin is thought to be fundamental in this process resulting in reduced adhesion of epithelial cells to desmosomes, increased cellular motility and dissemination of tumour cells into the circulation[4]. Once in the circulation tumour cells must home to a secondary environment where they will only be capable of forming metastases if the environment is appropriate[5]. On homing to bone it is thought that tumour cells occupy specific niches that are identical to, or overlapping with, the hematopoietic stem cell niche[6-7]. This niche is made up of two primary cell types: stromal cells and transient cells. Stromal cells include adipocytes, fibroblasts and osteoblasts and these originate from mesenchymal cells in the marrow. These cells contribute to the proliferation and differentiation of cancer cells via the secretion of molecules such as vascular cell adhesion molecule 1, syndecan-1 and matrix metalloproteinase 2 (MMP2)[8]. Transient cells include T cells, erythrocytes and platelets, all of which have been shown to stimulate tumour growth and metastasis[9]. Furthermore, the continuous process of bone remodelling, involving osteoclast mediated bone resorption resulting in the release of a multitude of growth factors, cytokines and cell adhesion molecules from the bone matrix making bone an attractive site for metastatic tumour cells[10-11].

The interactions between tumour cells and their microenvironment are important regulators of cancer metastasis and many excellent reviews have been published on this subject[10,12-13]. However, increasing evidence suggests that metastasis occurs as a result of a stepwise accumulation of genetic mutations, with different molecular alterations being required for different

stages in the metastatic process. In the current review we focus on the molecular alterations that drive the different stages of metastasis: tumour cell invasion and dissemination into the circulation, tumour cell homing to bone, and tumour cell colonisation and growth in the metastatic site (bone).

Tumour cell Invasion and dissemination into the circulation.

Amassing evidence shows that cells escaping primary tumours and becoming disseminated into the circulation have a mesenchymal phenotype and it is widely accepted that these cells originate from a subset of primary tumour cells that have undergone EMT. The exact molecular mechanisms that dictate EMT in tumour cells remain enigmatic, however studies of EMT that occur during developmental processes such as gastrulation and neural crest delamination provide clues as to how EMT may occur in metastasis[14]. Currently EMT is divided into three separate subtypes associated with distinct biological functions:

1) Implantation of embryogenesis and organ development.
2) Tissue regeneration and organ fibrosis and wound healing process,
3) Cancer progression and metastasis.

The induction of type 3 EMT at the onset of metastasis is facilitated by genetic alterations acquired by cancer cells and these generate cells with invasive properties that enable them to move into the blood stream (figure 1). In this review we will discuss the key transcriptional and cellular molecules that coordinate to drive EMT (as shown in figure 2). We will describe experimental evidence for new emerging concepts that challenge some current hypothesis to establish an up to date functional role for EMT mediators in the facilitation of tumour cell escape from the primary tumour and dissemination into the circulation.

The Transforming Growth Factor (TGF)-β superfamily

Members of the TGF-β superfamily including Bone Morphogenic Proteins (BMPs) and TGF-β, influence EMT via regulation of transcription factors SIP1,

slug and Snail which in turn supress the adhesion molecule E-cadherin causing loss of cell-cell adhesion[15].

Experimental evidence from murine mammary cancer 4T1 cells highlight the critical early role that TGF-β plays in inducing EMT. Cells treated with TGF-β acquired a spindle-like morphology and expression of the mesenchymal markers N-cadherin and vimentin. These changes were not seen when the cells were treated simultaneously with TGF-β and the TGF-β antagonist Schisandrin B (SchB). In addition, treatment of mice bearing 4T1 cells in a mammary fat pad with SchB resulted in significant suppression of metastasis to bone and lung but did not affect tumour growth at the primary site. Furthermore TGF-β appears to be specific to early events associated with tumour cell dissemination into the circulation as SchB did not inhibit lung metastasis when 4T1 cells were injected into the tail vein[16].

TGF-β activity is controlled by expression of BMP7 and up-regulation of BMP7 *in vitro* counteracts physiological EMT via inhibition of SMAD mediated TGF-β signalling and decreased expression of vimentin. In agreement with this BMP7 expression has been shown to be inversely proportional to the tumourgenisity and invasive behaviour of MDA-MB-231 breast cancer cells[17-18]. However, expression of BMP7 in primary tumour biopsies appears to contradict this finding. In a clinical setting, high levels of BMP7 expression in primary tumours has been strongly associated with accelerated bone metastasis, especially from ductal carcinomas[19]. Therefore the role of BMP7 in EMT remains inconclusive.

TWIST

The role of TWIST as a master regulator of type 1 EMT is well documented [20-22]. In addition to this there is a considerable amount of evidence suggesting that TWIST also plays a key role in type 3 EMT and metastasis[reviewed in 23-24]. TWIST is expressed in a variety of invasive and metastatic breast cancer cells including mouse 4T1 cells and human SUM1315, MDA-MB-231 and MDA-MB-435 cells. In contrast, this gene is not expressed on non-metastatic MDA-MB-436, MCF7 and BT20. siRNA knockdown of TWIST in 4T1 cells significantly reduces lung metastasis *in vivo* and numbers of tumour cells

shed into the circulation, without altering primary tumour growth[25]. The role of TWIST in stimulating EMT is further evident from experiments carried out in human mammary epithelial cells in which retroviral transfection with TWIST resulted in loss of cell-cell contact with elevated expression of vimentin, α-smooth muscle actin and N-cadherin as well as loss of E-cadherin, α-catenin, β-catenin and δ-catenin from the cell membrane[25]. Laboratory studies demonstrating TWIST activity induces metastasis has been extensively confirmed in clinical samples. Microarray analysis of different breast cancer subtypes revealed that TWIST is expresses by 70% of invasive lobular carcinomas and that tumour samples expressing TWIST also demonstrated other features of having undergone EMT including almost universal loss of E-cadherin (97%)[25]. Overall, data suggest that TWIST induces EMT by down-regulating E-cadherin mediated cell-cell adhesion in epithelial cells.

HIF-1α

It has been hypothesised that tumour cells undergo EMT in response to unfavourable conditions such as hypoxia in order to allow them to move to an environment that will better support their growth[26]. Evidence from *in vitro* studies using MCF7 cells show that hypoxia induced HIF-1α drives EMT and onset of metastasis via direct regulation of TWIST expression[27-28]. Under hypoxic conditions HIF-1α binds directly to the hypoxia response element of the TWIST promoter leading to increased expression of TWIST. This in turn results in upregulation of VEGF and the formation of new blood vessels, a subsequent increase in E-cadherin followed by loss of vimentin and N-cadherin. This process of hypoxia induced EMT can be completely reversed by inhibiting HIF-1α with siRNA implying that HIF-1α is a key regulator of EMT[27]. It therefore appears that hypoxia drives onset of metastasis by inducing HIF-1α and TWIST signalling arming the cells with a repertoire of molecules enabling escape from the local microenvironment into the circulation.

E-cadherin

Expression of E-cadherin has become a hallmark of many EMT assays and it is generally accepted that loss of this molecule is critical for the initial escape

and migration of individual cells from the primary tumour site[29]. E-cadherin is a single span transmembrane glycoprotein that interacts with Y-catenin in adjacent cells to form intercellular adhesion junctions[30]. Loss of E-cadherin disrupts cell-cell contact, leading to loss of the apical-basal barrier and cellular polarity, allowing growth factors that would normally have been segregated from the basolateral surface to interact with receptors in an autocrine fashion and further drive the EMT phenotype[31]. The critical role of E-cadherin in EMT and metastasis is well documented[2-3]. Reduced expression of this molecule in non-metastatic human mammary cell lines (HMLE) with shRNA results in loss of cell-to-cell contact and an increase in markers of mesenchymal cells. Furthermore, orthotopic injection of E-cadherin knockdown HMLE cells into the mammary fat pad of nude mice resulted in significantly more micro-and macro-metastases in the lungs compared with mice injected with control HMLE cells[32].

Loss of E-cadherin has generally been considered as being pivotal for the onset of metastasis, however, emerging evidence suggests that cell-to-cell contact is the critical key to metastasis and that disrupting this process can induce metastasis without altering E-cadherin. Plakoglobin is the gene that encodes the Y-catenin protein, which links E-cadherin to the actin cytoskeleton[33]. Knockdown of plakoglobin by miRNA in MCF7 and T47D breast cancer cells leads to loss of cell-cell contact and increased tumour cell invasion. Furthermore, implanting plakoglobin knockdown cells into mammary fat pads of nude mice resulted in increased shedding of tumour cells into the blood stream compared with animals injected with control cells[34]. Importantly, reduced plakoglobin expression resulted in an 80% reduction in Y-catenin, but did not alter E-cadherin expression or location of the cell membrane. Interestingly, however, decreased plakoglobin was found to reduce NM23-H1 (encoding nucleoside diphosphate kinase A, metastasis suppressor) that disrupts cell-cell adhesion via α-and β-catenin. Plakoglobin has been shown to promote α and β catenin nuclear translocation[34] implying that increased metastatic capability of cells can be driven independently of E-cadherin expression by loss of plakoglobin and NM23-H1.

Mucins

Aberrant expression of the transmembrane glycoproteins MUC1 and MUC4 a have all been associated with invasive epithelial carcinomas including breast. Recent evidence is emerging for a role of these proteins in EMT and metastasis. Experiments using an *in vitro* model of mouse mammary tumour cells (DA3) have demonstrated that overexpression of truncated human MUC1 leads to EMT, associated with ERK1/2 dependent activation of fibronectin and an increase in cell invasion[35]. Furthermore, in prostate cancer cells the cytoplasmic tail of MUC1 has been shown to interact with the DNA binding domain of androgen receptor, resulting in activation of EMT and increased invasiveness of cancer cells[36]. Direct evidence for an association between MUC1 and initiation of EMT in cancer has been shown in a mouse model of pancreatic ductal adenocarcinoma. Overexpression of MUC1 led to elevated levels of vimentin, slug and snail compared with levels in mutant MUC1 (MUC1 CT) mice. In addition, MUC1 in MUC CT mice did not immunoprecipitate with or cause nuclear translocation of β-catenin, thereby blocking the transcription of genes associated with EMT[37].

Studies utilising models of triple negative breast cancer have shown that MUC4 mucin enhances the invasive and migratory potential of cancer cells through upregulation of EGFR family proteins. A separate study demonstrated that knockdown of MUC4 in breast cancer cells leads to molecular and biochemical alterations that are necessary for EMT including reduced expression of mesenchymal markers such as vimentin and vitronectin and increased expression of the epithelial marker cytokeratin 18[38]. These data suggest that MUC4 plays an important role in EMT, transforming breast cancer cells into a more migratory and aggressive phenotype.

Cytokeratins 8, 18 and 19

Cytokeratins are the protein components of intermediate filaments that make up the supporting scaffolding within cells. The organisation and expression of these proteins change as epithelial cells gradually adopt an epithelial phenotype[39]. The change in expression profile of cytokeratins during EMT provides insight into how changes in structural integrity of cancer cells can drive invasion during cancer progression. In human breast cancer cells,

expression of cytokeratins K8 and K18 and 19 correlate with invasive potential, with high levels of K8 and K18 expressed in less invasive MCF7 compared with more invasive MDA-MB-231 and MDA-MB-436 cells and K19 being undetectable in invasive cells. Conversely, a negative correlation was seen between K8, K18 and K19 and filament proteins with increased vimentin and fibronectin observed in MDA-MB-231 and MDA-MB-436 compared with MCF7[40].

Direct evidence supporting an active role of cytokeratins in metastasis has come from a recent study that used shRNA to knock down K8 and K18 in epithelial cancer cells, hepatocellular carcinoma (EppG2) and cervical carcinoma (HeLa). Reduced expression of K8 and K18 in these cells led to hyperactivity of PI3K, NF-KB and Akt as well as increased expression of MMP2 and MMP9. Although it is recognised that K8 and K18 are involved in intracellular signalling and that these molecules are specific to epithelial cells, loss of these markers does not modify other markers of EMT. Instead, K8 and K18 are modulated by claudin-1 that influences the phenotype of epithelial cells independently of other EMT markers[41]. Therefore, it is likely that in addition to loss of K8 and K18 being a hallmark of EMT, modulation of these proteins at the transcriptional level influences cancer cell phenotype by orchestrating structural alterations that enable cells to escape from the primary tumour.

Fibronectin

The extra cellular matrix protein fibronectin plays a critical role in cellular adhesion, migration and transformation through activating the PI3/Akt pathway when bound to $\alpha_v\beta_1$ integrin[42]. Normal adult breast tissue is largely devoid of Fibronectin, however, during the process of tumourigenesis levels of this protein increase in the stroma with highest levels of expression detected at the invasive front of tumours during EMT[42]. *In vitro* evidence from human mammary breast cancer cells, MCF-10A, have highlighted a role for Fibronectin in promoting onset of EMT. Exposure of MCF-10A cells to exogenous Fibronectin stimulated cell migration and induced an EMT response including upregulation of the EMT markers Fibronectin Snail, N-

cadherin, vimentin, MMP2, α-smooth muscle actin and phospho-Smad2. In addition, exogenous Fibronectin is able to induce EMT under serum-free conditions; this process could be reversed following addition of a TGFβ neutralising antibody[43]. These data suggesting that Fibronectin is able to induce EMT in breast cancers via enhancing the activity of endogenous TGFβ.

Vimentin

The functional role of vimentin in EMT is yet to be fully elucidated, however, high levels of expression of this molecule are associated with an increased met static phenotype. Overexpression of vimentin in MCF7 breast cancer cells results in increased motility, whereas knocking down vimentin expression with siRNA in MDA-MB-231 cells down regulates genes associated with an invasive phenotype, Axl, ITGB4 and PLAU, and upregulates normal mammary epithelial genes, REAB25 and EHF[44]. Under non-cancerous conditions vimentin is involved in processes that require cell migration such as wound healing where it plays a pivotal role in determining cell polarity, regulation of cell to cell contact and transport of signalling proteins[45]. It therefore seems likely that altered expression of this molecule in cancerous tissue may also affect cell-cell adhesion and cell polarity enabling cells to detach from the primary tumour and invade the surrounding stroma.

Tumour cell homing to bone

Following degradation of the extracellular matrix tumour cells cross the basement membrane and enter the circulation cancer cells. Once in the blood and/or lymphatic systems tumour cells must home and disseminate to a secondary site where the microenvironment is favourable for them to proliferate and establish metastases. In theory, breast cancer cells can metastasize to any organ of the body, however, it has been demonstrated that breast cancer cells prefer to home to certain organs such as bone, lungs, liver and brain[5-6]. Even though the exact mechanism of the metastasis of breast cancer cells to these specific sites is still not completely understood. In 1889 Stephen Paget proposed that both cancer cells (seed) and the secondary

sites (soil) facilitate the process by releasing factors, signals and molecules that make the microenvironment appropriate and increase the probability of cancer cells to grow there (''seed and soil hypothesis'')[46], this hypothesis still appears to hold today and molecules involved in the process of bone homing are shown in figure 3.

Chemokines

One group of molecules that have been shown to play a crucial role in organ-specific metastasis of breast cancer cells are the chemokines and their receptors (G-protein couple receptors). The role of chemokines in organ-specific homing was first demonstrated in homing of lymphocytes and hematopoietic cells to different organs[47]. Studies have shown that breast cancer cells express these molecules leading to the hypothesis that they function as homing factors for breast cancer metastasis[48-49]. More specifically, the chemokine receptors CCR7 and CXCR4 were found to be upregulated in metastatic breast cancer cell lines as well as in primary breast tumours that metastasise[49]. Moreover, higher levels of their respective ligands CCL21/6Ckine and CXCL12/SDF-1a were found in sites that breast cancer cells preferentially migrate to such as bone, lung, liver and lymph nodes, compared with lower levels in small intestine and kidney which are sites that breast cancer cells rarely metastasise[49-50]. CXCR4 has been shown to specifically promote bone metastasis by stimulating tumour cell recruitment and homing to bone in response to its interaction with CXCL12/SDF-1a ligand[51]. Furthermore, inhibition of CXCL12/CXCR4 interaction using neutralizing antibodies or RNA resulted in reduced breast tumour growth as well reduced cell migration, invasion and metastasis[49, 52-53]. The role of CXCR4 in breast cancer cell homing to bone has further been demonstrated by genetic analysis of bone homing MDA-MB-231, non-bone homing MDA-MB-231 breast cancer cells and normal human mammary epithelium (MCF10A). In these experiments bone homing cells expressed significantly more CXCR4 compared with their parental clone and normal mammary epithelium and this overexpression was associated with higher number of bone metastases *in vivo*[2]. However, it should be noted that expression of CXCR4 is not a universal feature in breast cancer bone homing. We and

others have generated bone homing clones of MDA-MB-231 cells (BO2 and MDA-IV cells) using the same method as described by Kang et al[2,54-55]. Interestingly, CXCR4 was not detected in either of these bone homing cell lines by microarray or PCR analysis.

Intergrins

Intergrins are cell surface glycoproteins that facilitate cell-cell and cell-extracellular matrix adhesion and cell migration[Reviewed in 56]. Expression of these molecules on both tumour cells in bone and the supporting host stromal cells (osteoclasts, new blood vessels, inflammatory cells, platelets and bone marrow stromal cells) play key roles in enhancing bone metastasis. There are 8 beta and 18 alpha integrin subunits that can make up 24 different combinations in different cell types, each characterised by distinct ligand binding specificities, signalling abilities and regulatory mechanisms[Reviewed in 57]. Metastatic tumour cells show differential integrin heterodimerisation and activation compared with non-metastatic tumour cells that enable cells to home and colonise specific metastatic sites[58]. In bone metastasis the β1 and β3 family members appear to be of major importance.

In breast cancer αvβ3 is increased in expression in bone metastatic cells compared with non-metastatic cells[59]. Binding of αvβ3 integrin to bone extracellular matrix proteins including osteopontin, vitronectin and bone sialo-protein promote adhesion of breast cancer cells to bone[60]. Therefore it is likely that breast cancer cells expressing integrins that can interact with such proteins are more likely to home to bone. In 2002 Pecheur and co-workers showed that expression of αvβ3 integrin by breast cancer cells increased adhesion of cancer cells to bone and the incidence of bone metastasis[61]. Moreover, inhibition of αvβ3 integrin using a small molecule antagonist suppresses breast cancer bone metastasis indicating an explicit role for αvβ3 integrin in bone-specific homing[62]. The β1 family member, α5β1, specifically binds to fibronectin on human bone marrow stroma and expression of this molecule on breast cancer cells facilitates interaction with the bone stroma. The interaction between tumour cell α5β1 and host stromal cell fibronectin appears to contribute to the survival of growth arrested breast cancer cells, a

potential mechanism through which tumour cells can become sequestered and dormant within the bone marrow cavity and may later begin to proliferate and establish skeletal metastases[63].

Additional molecules associated with bone homing

In addition to chemokines and intergrins a multitude of other molecules have been shown to play important roles in chemotaxis of breast cancer cells to bone (see figure 2): Increased expression of MMP-1, interleukin 11 (IL-11) and connective tissue factor (CTGF) have been identified in bone homing clones of MDA-MB-231 breast cancer cells compared with parental cells *in vivo*[2]. Using a similar approach in which bone homing clones of MDA-MB-231 cells were compared with parental cells we and others have found that bone homong is associated with decreased cell-cell adhesion and migration, coupled with significantly reduced levels of the cell adhesion molecule fibronectin and calcium signal binding protein S100A4[54-55]. Interestingly, we also found a strong link between IL-1B expression and bone homing in both MDA-MB-231 cells and primary tumours from breast cancer patients indicating that this molecule may promote an invasive and motile phenotype in breast cancer cells[55].

Tumour cell colonisation and growth to bone

Tumour cells are disseminated into the bone environment via the blood stream and as a result the most common homing site is the highly vascularised metaphysis in the long bones. Once in the bone microenvironment it is hypothesised that breast cancer cells compete for the hematopoietic stem cell (HSCs) niche and once within this niche they can be stimulated to proliferate[55, 64]. In addition to molecules with well defined roles in bone colonisation, discussed below, we have previously demonstrated that this process is also associated with increased levels of MMP9, HRAS and fibronectin[55]. MMP9 has strong associations with increased tumour cell invasion in a number of cancer types[65] and increased MMP9 expression has

been shown to increase the capacity of tumour cells to extravasate as well as to cause activation of bone resorbing osteoclasts[66-67]. In addition to the pro invasive properties of MMP9 HRAS over-expression has been shown to increase invasion of MCF10A breast cells and to transform these from an endothelial to an epithelial cell type (MET)[68]. Fibronectin on the other hand has well characterised properties as a cell adhesion molecule and has been shown to be upregulated in bone metastatic deposits compared with primary breast tumours[69]. It is therefore likely that increased expression of fibronectin enables tumour cells to adhere to the HSC niche once they have successfully entered the bone microenvironment[60, 70].

Mesenchymal to Epithelial transition and bone colonisation

To enable colonisation of bone, tumour cells undergo a reverse EMT transition known as Mesenchymal-Epithelial transition (MET), reactivating their epithelial cell properties and increasing their adhesion and interactions with the bone marrow microenvironment[71-72] (Figure 3). The re-expression of E-cadherin is considered the fundamental hallmark of MET, as it allows tumour cells to interact with the bone marrow and adhere to the HSC niche[31, 71]. Once in the bone, tumour cells first form micro-metastases that can either proliferate and form overt metastatic lesions or remain dormant for long periods until reactivate and establish tumours[73]. The mechanisms by which some tumour cells remain dormant whilst others are stimulated to proliferate remain to be established. However, amassing evidence suggests that factors that increase bone turnover including menopause / ovariectomy may stimulate the cells within the mesenchymal stem cell niche and drive proliferation of dormant tumour cells[74]. Increased proliferation of tumour cells in the bone marrow causes the production of molecules and growth factors by both tumour cells and bone resorption that promote tumour growth, osteoclasts production, osteoblasts inactivation and bone destruction (osteolysis), a process known as ‘’vicious cycle’’ as shown in figure 3[73]. More specifically, proliferating cancer cells have been shown to release factors such as parathyroid hormone-related protein (PTHrP), interleukins (IL-8,11), MMP-1, cyclooxygenase-2 (COX-2), transcription factor GLI2 and hypoxia-induced

growth factor 1 α (HIF1α) that promote both their growth in the bone marrow and osteolysis.

PTHrP

PTHrP is a major regulator of osteolytic lesion formation and a key molecule responsible for the humoral hypercalcemia of malignancy[75-76]. Moreover, PTHrP has been shown to be expressed in 92% of primary breast cancers that metastasised to bone compared with tumours that metastasised to non-bone sites (17%) and primary tumours that did not metastasise (60%)[77]. It is hypothesised that release of PTHrP by tumour cells primes the bone environment for growth of metastatic tumour cells by increasing bone turnover. Release of transforming growth factor-β (TGF-β) from the bone marrow increases PTHrP secretion from breast cancer cells via activation of Smad and p38 MAP kinase pathways[78]. This stimulates the production of receptor activator of nuclear factor-κB ligand (RANKL) that subsequently binds to RANK and induces osteoclast differentiation and activation[79]. Under normal physiological conditions, the binding of RANK to RANKL is homeostatically controlled by binding of RANK to it's decoy receptor, soluble ligand, osteoprotegerin (OPG) (Simonet et al., 1997). However, production of PTHrP by tumour cells has been shown to inhibit OPG activity thus promoting bone metastasis (Chiechi et al., 2013). Breast cancer cells also express cyclooxygenase-2 (COX-2) which plays an important role in the development of osteolytic bone metastasis by driving over-expression of prostaglandin E2 (PGE2)[80]. Moreover, the production of osteolytic factors including IL-8 and IL-11 by breast cancer cells appear to play an important role in osteoclast formation. Bendre and co-workers showed a direct effect of IL-8 in osteoclast differentiation and activation leading to the knock on effect of bone destruction[81]. In addition, it was shown that IL-11 mediates bone resorption by increasing the osteoblast production of RANKL[2]. Evidence from IL-11 overexpressing cell lines indicate that this molecule also has direct effects on promoting bone metastasis *in vivo*[82]. Similarly to IL-11, MMP-1 promotes osteolytic bone metastasis by increasing the osteoblast production of RANKL and also by suppressing the expression of osteoprotegerin through activation of EGFR-dependent signalling pathway[83]. Metastatic breast cancer cells often

express HIF1α that promotes osteoclast formation and inhibit osteoblast differentiation (activation)[84] as well as transcription factor GLI2 that promote the production of PTHrP, facilitating osteolysis[85].

TGF-β

As a result of bone destruction stimulated by the osteoclast promoting factors released from breast cancer cells, growth factors such as TGF-β stored in a latent form in bone marrow are released[86]. The activation of TGF-β signalling has been observed in several studies and correlates with breast cancer bone metastasis formation[87-88]. It therefore appears that breaking the vicious cycle between tumour growth and bone destruction may be necessary for successful treatment of breast cancer bone metastasis. In an attempt to address this, a small molecule targeted against the TGF-β receptor 1 has been developed. YR-290 blocks TGF-β signalling by inhibiting nuclear translocation of Smad 2 and reducing phosphorylation of Smad 2/3. Treatment of HaCaT cells with YR-290 inhibits TGF-β-induced EMT, resulting in dose-dependent increases in expression of vimentin and fibronectin and in increase in E-cadherin. Furthermore, in a 4T1 mouse model, YR-290 blocked spontaneous metastasis to lung by 74.71% and 97.38% in groups treated with 1mg/kg/day and 5mg/kg/day, respectively. Moreover, it has been demonstrated that blocking of TGF-β signalling in MDA-MB-231 breast cancer cells inhibited the development of osteolytic bone metastases *in vivo*[89]. Although these data are from early stage experiments they provide an incite into the potential therapeutic benefits of targeting EMT/MET to produce noval treatments for preventing development and progression of breast cancer bone metastasis.

Conclusions

The molecular alterations associated with driving breast cancer metastasis to bone are still not fully understood, however, it is clear that this involves a vast number of molecules leading to large scale functional and architectural change of the cancer cell. Metastasis appears to be primarily driven by alterations in adhesion complexes and the cytoskeleton enabling the cell to firstly escape from the primary tumour and then lodge in its metastatic site.

The exact chronology of individual changes remains unclear and a number of molecular pathways converge to generate an altered phenotype. The switch between one phenotype and another is rapid and the exact order in which these happen may prove difficult to identify. Furthermore, it is still unclear which, if any, of these molecules has the overriding influence on EMT/MET. Being a trigger of EMT is a claim common to TGF-β, TWIST, HIF-1α and E-Cadherin with alterations in the remaining molecules having an effect on EMT to differing extents. Redundancy that is typical in biological systems, may prove to be problematic when choosing a suitable EMT/MET related target for blocking the spread of cancer. However, as seeding of breast cancer cells into the bone is an early event it is likely that tumour cells may already be present in the bones of patients before they are diagnosed with breast cancer[90-91]. Therefore, molecules associated with early onset of metastasis may provide useful biomarkers for predicting disease recurrence. For drug targets, it may be more pertinent to identify novel molecules associated with tumour growth in bone.

Conflicts of interest

Authors have no conflicts of interest related to this article.

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Figure legends

Figure 1. Breast cancer metastasis is initiated by tumour cell dissemination into the circulation. Diagrammatic representation showing the molecular alterations that drive phenotypic alterations in cancer cells leading to tumour cell dissemination into the blood and lymphatic vesels.

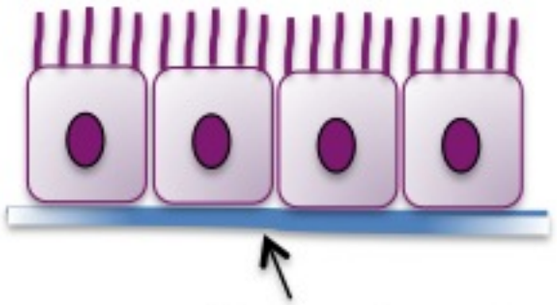
Figure 2. Molecular pathways involved in epithelial to mesenchymal transition. Molecules shown in red are downregulated and molecules shown in green are upregulated during epithelial to mesenchymal transition.

Figure 3. Breast cancer cells home to the bone microenvironment. Bone is made up of different cell types that express a variety of molecules including the chemokine CXCL12 that attract breast cancer cells to this microenvironment. Breast cancer cells also express molecules including the chemokine receptor CXCR4 that enable them to adhere to the osteoblast rich HSC cell niche and disseminate in the bone. Once in the bone microenvironment tumour derived growth factors prime the bone for subsequent colonisation of tumour cells.

Figure 4. Breast cancer cells disseminated in bone colonise this environment. Disseminated breast cancer cells in the bone microenvironment undergo mesenchymal to epithelial transition enabling colonisation and growth at this metastatic site. Tumour cells growing in bone secrete growth factors that stimulate osteoclastic bone resorption. This in turn result in a vicious cycle whereby osteoclasts release bone derived growth factors that further stimulate tumour growth.

Figure 1

Normal epithelium

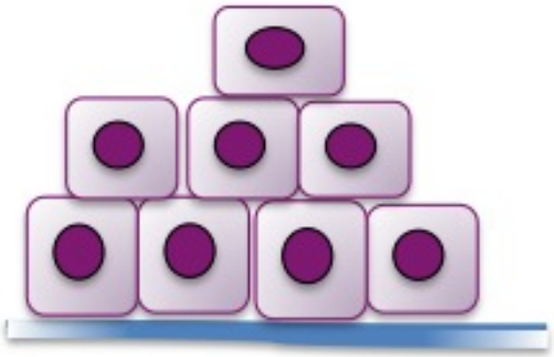


Basement membrane

↑proliferation

↓apoptosis

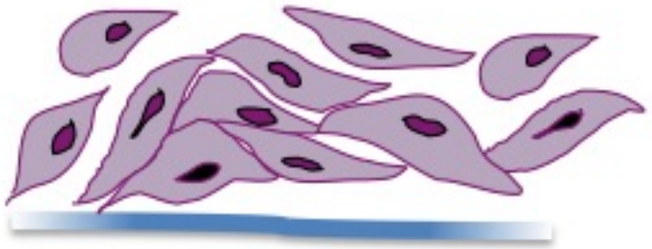
Dysplasia



↑TGFβ
sip1
slug
snail
twist
hif-1α

↓E-cadherin
β-cadherin
α-cateinin
f actin

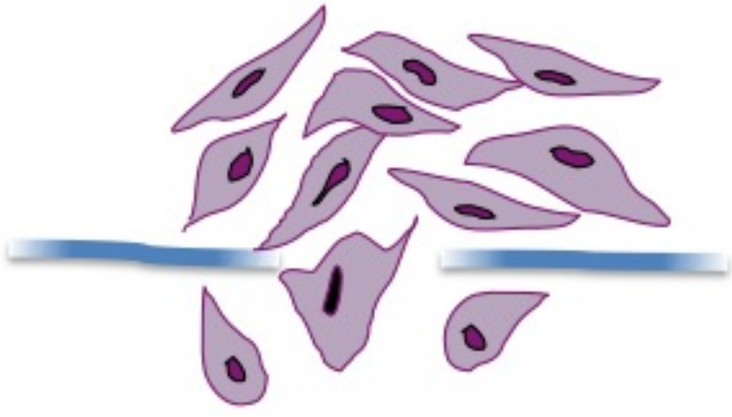
Loss of adhesion:
Intercellular and basement membrane



↑vimentin
muc1
muc4

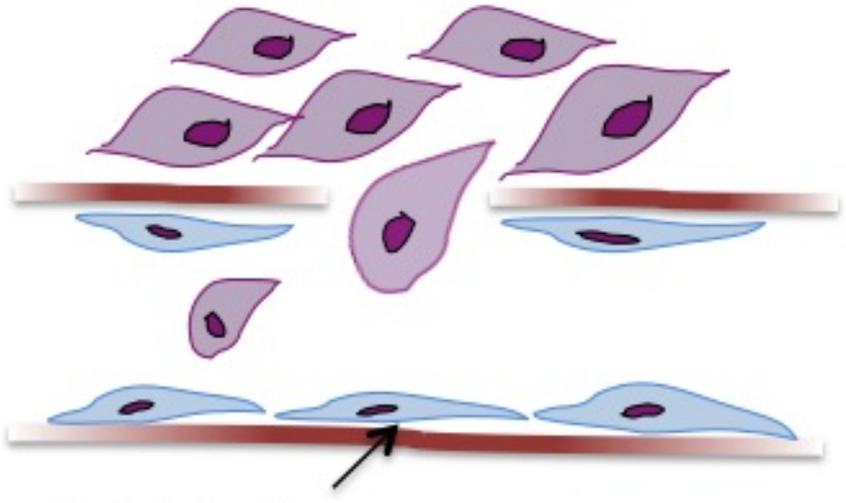
↓ϒ-catenin
desmoplakin
occudin
cytokeratins

Invasion of basement membrane and migration into the surrounding stroma



↑N-Cadherin
slug
snail
twist
FSP-1
α-SMA
vimentin
mucins

Intravasation



Endothelial cells

Figure 2

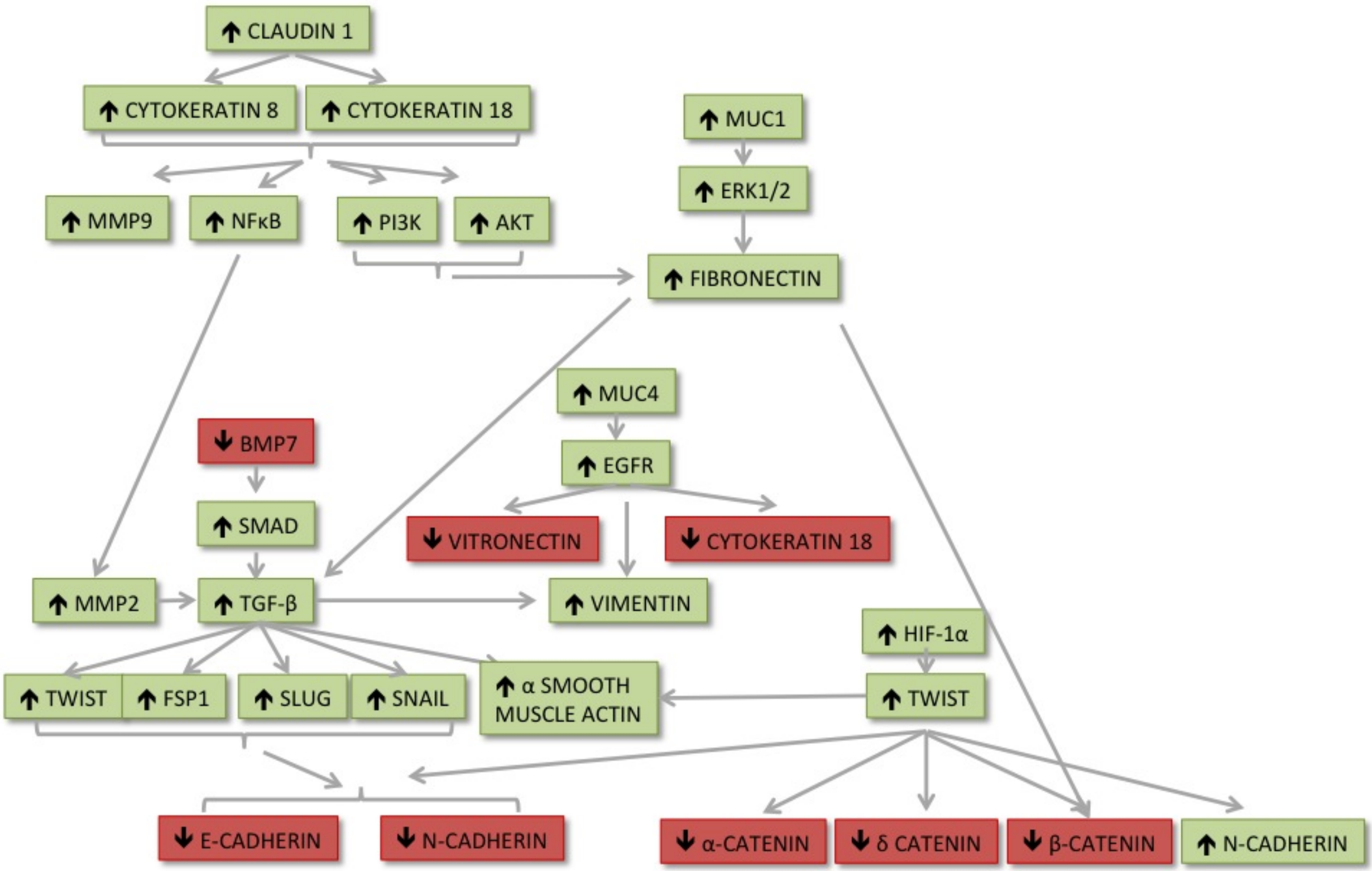

↑ CLAUDIN 1
↑ CYTOKERATIN 8
↑ CYTOKERATIN 18
↑ MMP9
↑ NFκB
↑ PI3K
↑ AKT
↑ MUC1
↑ ERK1/2
↑ FIBRONECTIN
↑ MUC4
↓ BMP7
↑ EGFR
↑ SMAD
↓ VITRONECTIN
↓ CYTOKERATIN 18
↑ MMP2
↑ TGF-β
↑ VIMENTIN
↑ HIF-1α
↑ TWIST
↑ FSP1
↑ SLUG
↑ SNAIL
↑ α SMOOTH MUSCLE ACTIN
↑ TWIST
↓ E-CADHERIN
↓ N-CADHERIN
↓ α-CATENIN
↓ δ CATENIN
↓ β-CATENIN
↑ N-CADHERIN

Figure 3

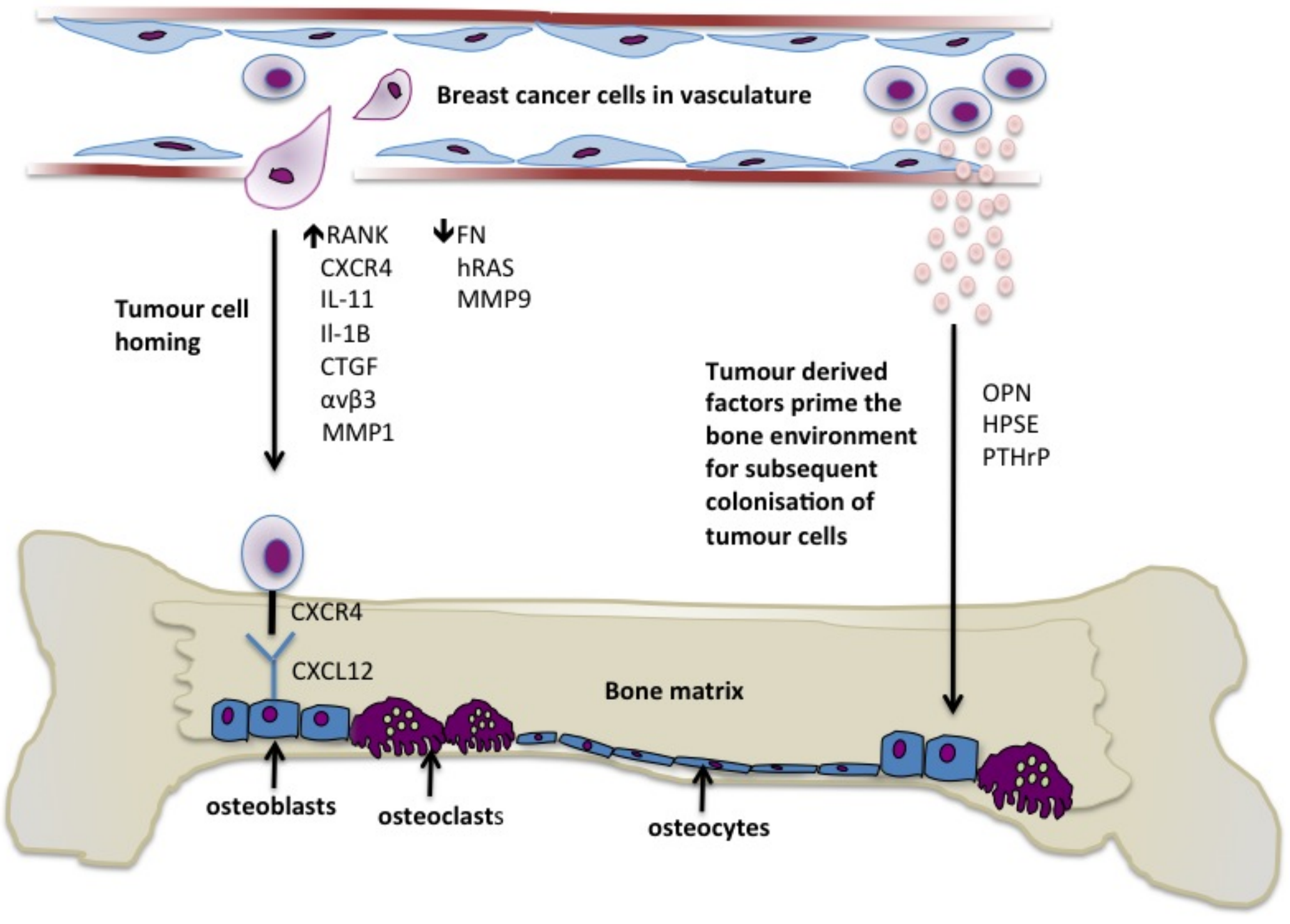
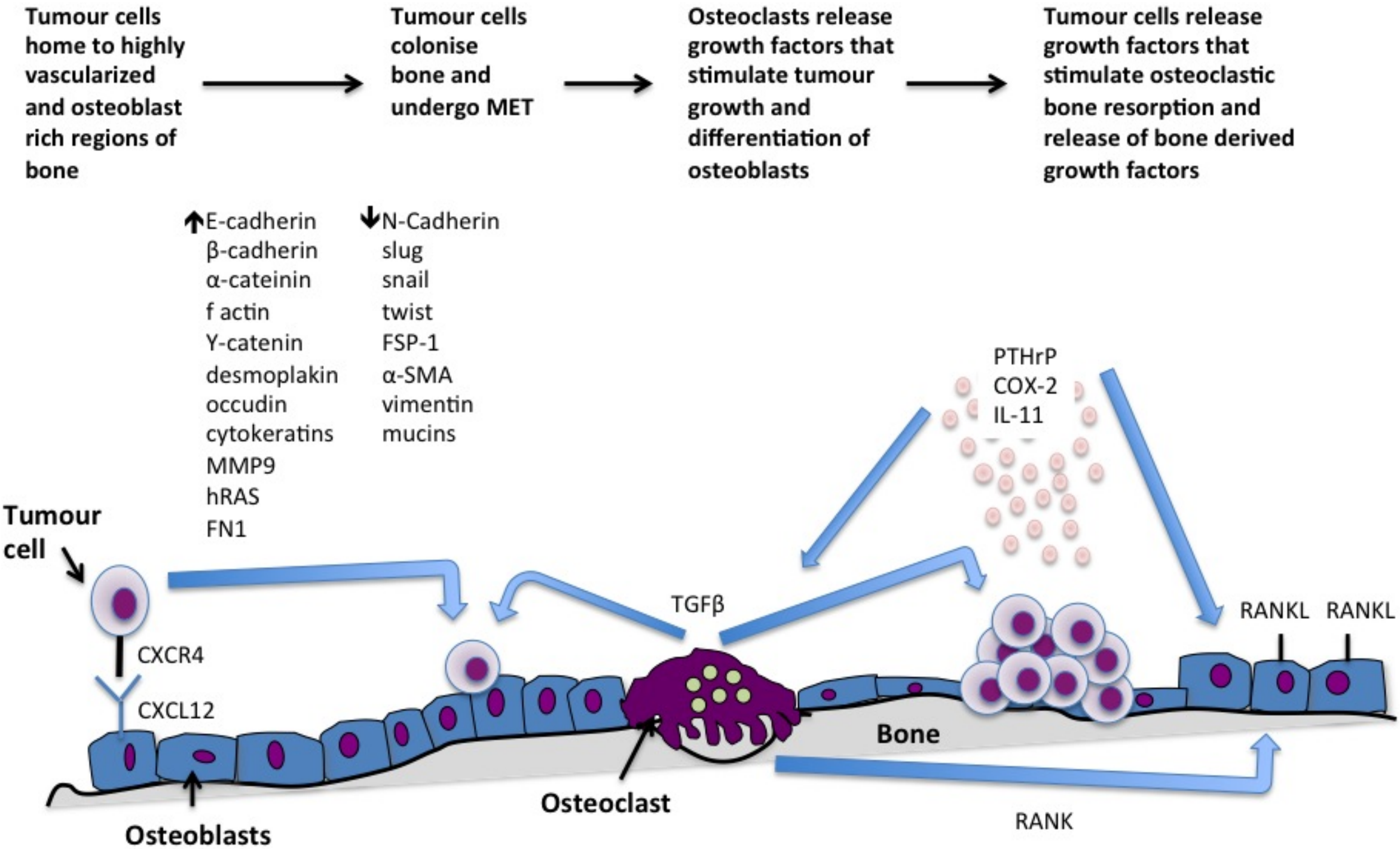

Breast cancer cells in vasculature
Tumour cell homing
↑RANK
CXCR4
IL-11
Il-1B
CTGF
αvβ3
MMP1
↓FN
hRAS
MMP9
Tumour derived factors prime the bone environment for subsequent colonisation of tumour cells
OPN
HPSE
PTHrP
CXCR4
CXCL12
Bone matrix
osteoblasts
osteoclasts
osteocytes

Figure 4


Tumour cells home to highly vascularized and osteoblast rich regions of bone
Tumour cells colonise bone and undergo MET
Osteoclasts release growth factors that stimulate tumour growth and differentiation of osteoblasts
Tumour cells release growth factors that stimulate osteoclastic bone resorption and release of bone derived growth factors
↑E-cadherin
β-cadherin
α-cateinin
f actin
Y-catenin
desmoplakin
occudin
cytokeratins
MMP9
hRAS
FN1
↓N-Cadherin
slug
snail
twist
FSP-1
α-SMA
vimentin
mucins
PTHrP
COX-2
IL-11
Tumour cell
CXCR4
CXCL12
TGFβ
RANKL
RANKL
Bone
Osteoclast
Osteoblasts
RANK