Review

Molecular Mechanisms of Bone Metastasis

ULRICH H. WEIDLE, FABIAN BIRZELE, GWENDLYN KOLLMORGEN and RÜDIGER RÜGER

Roche Pharma Research and Early Development, Roche Innovation Center Penzberg,
Roche Diagnostics GmbH, Penzberg, Germany

Abstract. *Metastasis of breast and prostate cancer as well as multiple myeloma to the bones represents a significant medical problem. We herein discuss the molecular basis of the creation of pre-metastatic niches, the process of bone metastasis and the phenomenon of tumor dormancy in the bone marrow as well as its regulation. We describe the identification and validation of genes mediating bone metastasis by use of pre-clinical models of bone metastasis. Additionally, we discuss the role of small integrin binding N-linked glycoproteins (SIBLINGS), the chemokine/chemokine receptor CXCL12/CXCR4 pathway and the role of micro RNAs (miRNAs) as mediators of bone metastasis. Finally, we summarize clinical achievements for the treatment of bone metastases.*

Preferential distant organs for metastasis of tumors are the lung, liver, brain and bone (1). Prostate, lung, breast, kidney and thyroid cancer account for 80% of skeletal metastases (2). Multiple myeloma has also a strong preference for growth and metastasis in the bone marrow. The most common sites of bone metastases are the spine, ribs, pelvis, proximal femur and skull. Breast cancer preferentially metastasizes to the lungs and bones, whereas prostate cancer almost exclusively metastasizes to the bones (3). Bone metastases can be classified as osteolytic with significant bone destruction, osteoblastic due to excess bone formation or a mixed phenotype can occur (4, 5). Metastasis to the bones is facilitated by the fenestrated structure of the bone marrow sinusoid capillaries, high blood flow in the areas of red marrow and adhesive molecules on tumor cells that bind to the bone marrow stromal cells such as osteoblasts and

osteoclasts as well as the bone matrix (6, 7). These cell types play an important role in bone re-modeling and niche structure (8-10).

The bone marrow microenvironment includes osteoblastic (endosteal) and vascular niches, that provide an environment supporting hematopoietic and non-hematopoietic stem cells (11). Bone homeostasis is maintained by balanced production of osteoblasts and osteoclasts. Disruption of this balance can convert normal niches into metastatic niches. Disseminated tumor cells in the bone marrow can persist in a dormant state for many years until they progress to macrometastatic lesions (12). The clinical challenges associated with bone metastasis are deleterious effects such as bone pain, fractures, life-threatening hypercalcemia, spinal cord compression and other nerve compression syndromes (13). Deeper understanding of the process of bone metastasis and identification of targets for prevention and treatment is, therefore, an objective of paramount importance. In the following we describe the process of metastasis to the bones, the role of interaction of tumor cells with stromal cells in this context and potential targets which are involved in bone metastasis. We also discuss current and emerging treatment modalities for bone metastasis.

Bone metastases. Osteblasts and osteoclasts are crucial for interactions with tumor cells in the context of their colonization of the bone marrow. Osteoclasts are multi-nucleated cells that arise from precursor cells of the monocyte-macrophage lineage. They de-mineralize bone due to dissolving calcium phosphate crystals by producing acid and are involved in bone resorption through degradation of the newly-exposed extracellular matrix (ECM) by secreted proteases. Osteoclasts adhere to bone surface and the spectrum of factors involved in their activation may depend on tumor type. Osteoblasts are specialized, terminally differentiated mononuclear cells differentiated from mesenchymal stem cells and are involved in bone reconstruction by generation of new ECM and deposition of calcium phosphate crystals into the interstices of the matrix (14). Prerequisite for the generation

Correspondence to: Rüdiger Rüger, Roche Innovation Center, Im Nonnenwald 2, D-82372 Penzberg, Germany. Tel: +49 885660 2022, e-mail: ruediger.rueger@roche.com
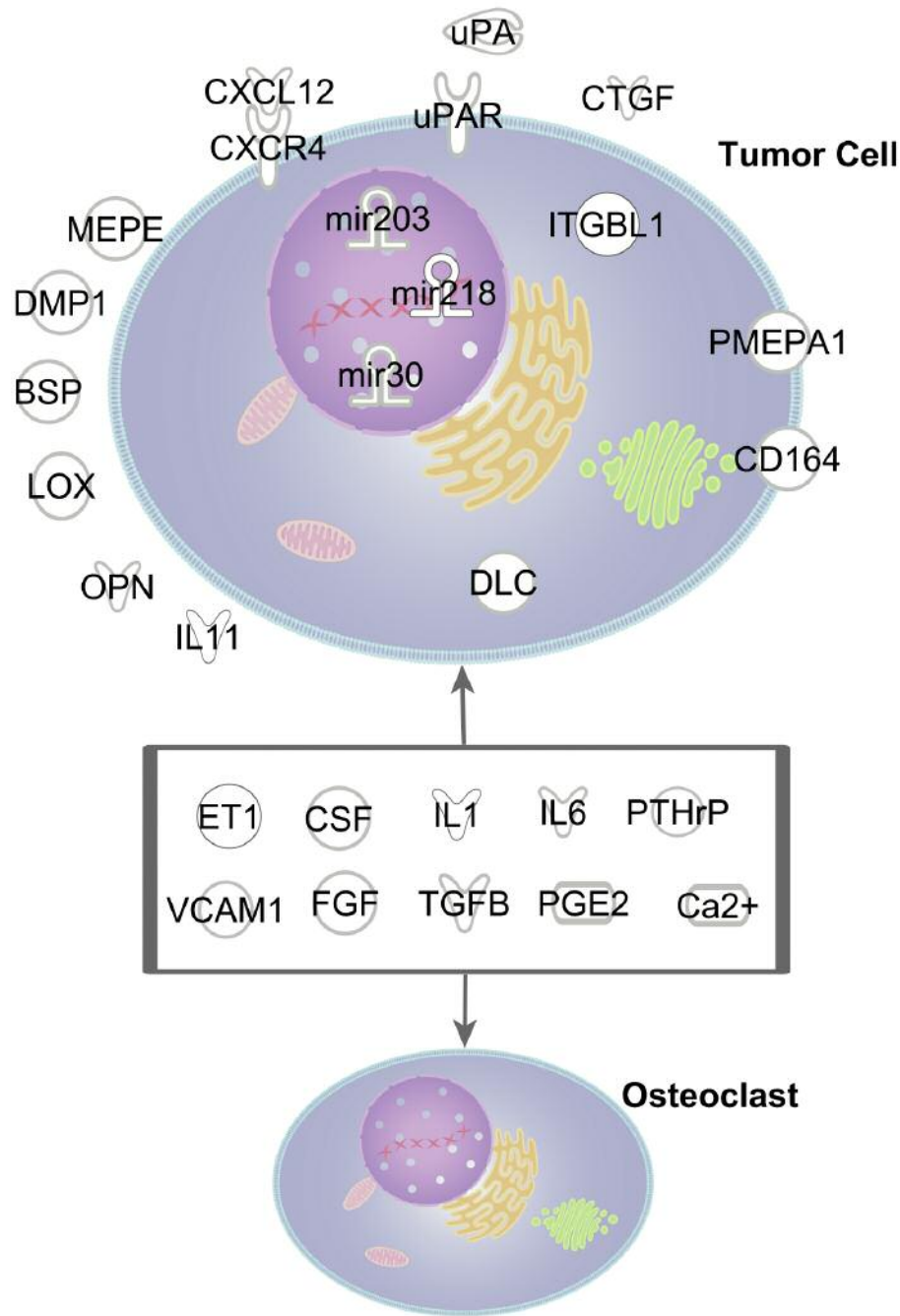
of osteolytic metastases is the activation of osteoclasts. To this end, osteoblasts secrete receptor activator of NF-κB ligand (RANKL), which interacts with osteoclast precursors displaying RANK receptor on their surface, resulting in their activation and finally maturation into functional osteoclasts. Osteoblasts also produce osteoprotegerin (OPG), a soluble decoy receptor which can block RANK/RANKL signaling by scavenging of RANKL. Thus, the activation of osteoclasts is triggered by the balance between RANKL and OPG (15, 16). RANKL also can induce factors involved in migration, invasion and angiogenesis such as matrix metalloproteinases 1 and 9 (MMP1, MMP9), matrix metalloproteinase inducer EMMPRIN/CD147, intercellular adhesion molecule-1 (ICAM-1), interleukin 6 and 8 (IL6, IL8) and vascular endothelial growth factor (VEGF) (17) and decrease the expression of metastasis suppressor serpin 5b/maspin (18). RANKL can also promote the function of regulatory T-cells (Tregs) and macrophages (19, 20). Osteolysis is based on a self-perpetuating signaling system (vicious cycle) that is maintained by mitogenic factors for tumor cells such as transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs) and Ca-ions released from demineralized bone as well as parathyroid hormone-related peptide (PTHrP) derived from tumor cells. PTHrP acts as a promotor of osteolysis by osteoclasts (21).

Breast cancer-derived bone metastases are osteoclastic. Mediators of survival of osteoclast progenitors and activation factors of osteoclasts, such as interleukin 1 (IL1), interleukin 6 (IL6), PTHrP, prostaglandin E2, colony-stimulating factor-1 (CSF-1) and tumor necrosis factor-α (TNFα) have been identified for this type of tumor (22). A bone metastasis suppressive pathway was identified using a breast cancer *in vivo* system (23). In this pathway, Deleted in liver cancer 1 (DLC1) was delineated as the mediator of metastasis suppression. DLC1 acts as a RhoGTPase activating protein (RhoGAP) which inhibits RhoA, RhoB, RhoC and cell division cycle 42 (cdc42) by stimulating the hydrolysis of GTPase bound GTP. It was shown that DLC1-Rho signaling regulates osteoclastogenesis by blocking TGFβ-induced PTHrP secretion, a mediator of osteoclast maturation, as a prerequisite for osteolytic colonization. Interestingly, the DLC1-Rho pathway regulates metastatic colonization of circulating breast cancer cells in bone, not in the lungs, since it has been noted that silencing of DLC1 is associated with poor prognosis in breast cancer patients (23).

Additionally, lung cancer-derived bone metastases are predominantly osteolytic. A distinct cytokine profile based on IL1, IL7, PTHrP and RANKL, associated with this process, has been identified (24). Osteoblastic bone metastases are preferentially associated with prostate cancer. Osteoblasts are activated by prostate cancer cells and the tumor microenvironment leading to accumulation of immature mineralized bone (osteoid) in the vicinity of metastases (25, 26). Osteonectin, TGFβ, CXC chemokine ligand 12 (CXCL12) and VEGF were shown to be involved in extravasation and guidance of prostate cancer cells to the bone and an important role of cadherin 11 in binding of prostate cancer cells to osteoblasts was noted (27). Bone morphogenetic protein (BMP), endothelin-1 (ET-1) and PDGF secreted by tumor cells as well as Wnt signaling were identified as mediators of the process of generation of osteoblastic metastases (28). An N-terminal fragment derived from urokinase can act as a potent stimulator of proliferation of prostate cancer cells (29). A survival and proliferation-mediating role for prostate cancer cells was assigned to FGFs, IGF and TGFβ, all originating from the tumor microenvironment (30). Prostate-specific antigen (PSA), a kallikrein-type serine protease, was shown to cleave and activate PTHrP, thus accelerating growth of bone metastases (31). A selection of targets discussed in this review is shown in Figure 1.

Pre-metastatic niches in the bone marrow. Niches in the bone marrow can be newly-induced as a result of tumor-secreted factors or they can be adapted from pre-existing physiological niches such as stem cell niches. Interaction between activated stromal cells and other cells in the pre-metastatic niche enables survival of tumor cells. Bone marrow-derived cells and other stromal cells are involved in the creation of the pre-metastatic niche before arrival of disseminated tumor cells (32-35). Pre-metastatic niche-promoting factors such as human S100 calcium binding proteins A8 and A9 (S100A8/9), serum amyloid A3 (SAA3), CXCL12, TNFα, TGFβ, MMPs, secreted fibronectin, VEGF-A and placental growth factor (PlGF) have been identified (32-35). They can be derived from different cell types such as hematopoietic progenitor cells (HPC), mesenchymal cells, platelets, endothelial progenitor cells (EPC) and fibroblasts. As a next step, micrometastases are formed by recruitment of metastatic tumor cells into the pre-metastatic niche. Bone micrometastasis can stay in a stem-like, dormant state before expansion into overt metastases. In addition to factors as outlined above, P- and E-cadherin are involved in adhesion and extravasation of metastatic tumor cells, cell-cell interactions mediated by ligation of cluster of differentiation 44 (CD44) and generation of cross-linked extracellular matrix by lysyl-oxidase (LOX) promote survival of metastatic tumor cells in the metastatic niche. Finally, the macrometastatic stage of the niche is induced by EPCs that are responsible for an angiogenic switch mediated by factors such as VEGF-A and PlGF. In case of bone metastases, tumor-associated myeloid cells and stromal cells such as fibroblasts, osteoclasts and osteoblasts are involved in this process. Genesis of an early bone metastatic niche was investigated in a breast cancer micrometastasis model after

Figure 1. *Overview of selected targets mediating bone tropism of metastasis. The upper part of the figure shows tumor cell-related targets. These comprise of intracellular targets, membrane receptors, components of the extracellular matrix and secreted factors. The box in the middle part shows factors derived from tumor cells as well as from osteoclasts which interact with both cell types resulting in a vicious cycle. The lower part shows a schematic representation of an osteoclast. Ingenuity pathway analysis (IPA) was used for designing the figure. BSP, Bone sialoprotein; CD164, cluster of differentiation 164; CSF, colony-stimulating factor; CXCL12, CXC chemokine ligand 12; CXCR4, CXC chemokine receptor 4; CTGF, connective tissue growth factor; DMP1, dentin matrix protein 1; DLC, deleted in liver cancer; ET1, endothelin 1; FGF, fibroblast growth factor; IL1,6,11, interleukin 1,6,11; ITGBL1, integrin β-like 1; LOX, lysyl-oxidase; miR 30, 203, 218, microRNA 30, 203, 218; MEPE, matrix extracellular phosphoprotein; OPN, osteopontin; PGE2, prostaglandin E2; PMEPA-1, prostate transmembrane protein, androgen induced 1; PTHrP, parathyroid hormone related protein; uPA, urokinase plasminogen activator; uPAR, urokinase receptor; VCAM-1, vascular cell adhesion molecule.*

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injection of tumor cells and stromal cells into the iliac artery of mice (36). *In vivo* co-injection experiments of tumor cells and osteogenic mesenchymal stromal cells indicated that stromal cells promote tumor cell proliferation in a contact-dependent manner. In this model, the microenvironment is composed of cells abundantly secreting alkaline phosphatase and collagen-1 with low abundance of osteoclasts. Mechanistic studies have shown that heterotypic adherens junctions between E-cadherin expressed on the surface of tumor cells and N-cadherin on the surface of osteogenic cells lead to activation of mammalian target of rapamycin (mTOR) and AKT signaling and finally to recruitment and activation of osteoclasts. RNA-based signatures derived from breast cancers confirm enhanced mTOR activity in correlation to bone metastatic progression (37). mTOR signaling has been shown to be implicated in the resistance of ER$^+$ breast cancer to endocrine therapy (38), pointing to a possible role of metastatic niches in this context. Systemic induction of a metastatic niche in bone by the ECM-modifying enzyme LOX secreted by hypoxic ER-negative breast tumor cells was demonstrated (39). Making use of a transplantable breast cancer model, it was shown that secreted LOX regulates bone homeostasis *via* osteoclastogenesis. LOX-mediated disruption of bone homeostasis is driven by nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) and is independent of RANKL. The formation of pre-metastatic lesions can act as a platform for circulating tumor cells (CTC) to colonize and form metastases. High expression of LOX in primary tumors or systemic delivery of LOX resulted in osteolytic lesion formation that could be inhibited by silencing or inhibition of LOX with small molecules. Retrospective analysis of lymph node-negative breast cancer patients has indicated that a hypoxic signature and expression of LOX are indicative of bone metastases in patients with ER-negative tumors, not in those with ER-positive tumors. These findings might be relevant for stratification of breast cancer patients with a high risk for bone metastasis and inhibition of LOX to prevent bone metastasis. Drugs that prevent bone metastasis such as bisphosphonates and denosumab (40) might have an impact as efficient co-therapies for prevention of bone metastasis. In addition, it should be mentioned that involvement of exosomes in the generation and promotion of metastatic niches has been described by several groups (41-43).

Disseminated tumor cells in the bone marrow. Disseminated cells originating from breast, prostate and lung tumors can be frequently detected in the bone marrow and quantified and characterized with established methodology (44). Extended time of latency at pre-metastatic sites, which may last for several years, has been noted for breast and prostate cancers (45). The clinical relevance of these findings is emphasized by the fact that the abundance of tumor cells in bone marrow (BM) aspirates correlates with reduced metastasis-free survival (46).

For breast cancer, a preference of ER-positive tumors for homing to the BM has been noted (47, 48). Therefore, ER antagonists may prevent the growth of micrometastases. The observed dormancy is probably due to delayed adaption of the tumor cells to the foreign microenvironment. Dormancy is characterized by a balance between apoptosis and proliferation and the inability to recruit a vascular bed and to overcome immunosurveillance (12). It was demonstrated that circulating prostate tumor cells can occupy the niche for hematopoietic stem cells (HSC) after their arrival in the BM and are able to adapt to their hypoxic conditions (49). A switch from a mesenchymal to an epithelial phenotype has been noted in the context of homing of tumor cells to the BM (50, 51). Dormancy can be recapitulated in experimental models after resection of the primary tumor, creating a scenario which mimics the situation in patients (52). Disseminated tumor cells (DTC) in the BM which lodge into the HSC niche maintain a cancer stem cell state (CD44$^+$, CD24$^-$) (12). For breast cancer, a src signature correlates with relapse to the bones, but not to other organs (53). CXCL12/CXCR4 interaction has been shown to activate src and promote survival of DTC in the BM (53). Osteogenic cells play a crucial role in the re-activation of dormant tumor cells, by secretion of IL11 they mediate the activation of vascular cell adhesion molecule 1 (VCAM1) expression on tumor cells and further recruitment of osteoclasts by interaction with integrin-α4β1 on their surface (54-56). Additional mechanisms due to interaction of tumor cells and stromal cells resulting in re-activation of dormant tumor cells and promoting their survival as well as conversion of preosteoblasts to osteoblasts have been identified and is outlined in the following. TGF-β has been shown to induce Notch-ligand jagged in tumor cells which promotes secretion of IL6 by osteoblasts, increasing tumor cell survival and proliferation as well as conversion of preosteoclasts into osteoclasts (55). Activation of sympathetic neuron signaling by BM stromal cells increases RANKL production by osteoblasts and promotes formation of osteolytic metastasis (57). Also, a shift in the balance between extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase p38 due to stress signaling was shown to promote re-activation of dormant tumor cells (58).

SIBLINGS

Small integrin binding ligand N-linked glycoproteins (SIBLINGS) are soluble glyco-phosphoproteins that in normal tissues mediate cell adhesion, motility and survival as signal transducers after binding to integrins and CD44 (59). Members of the SIBLING family are osteopontin (OPN), bone sialoprotein (BSP), dentin matrix protein 1 (DMP1),
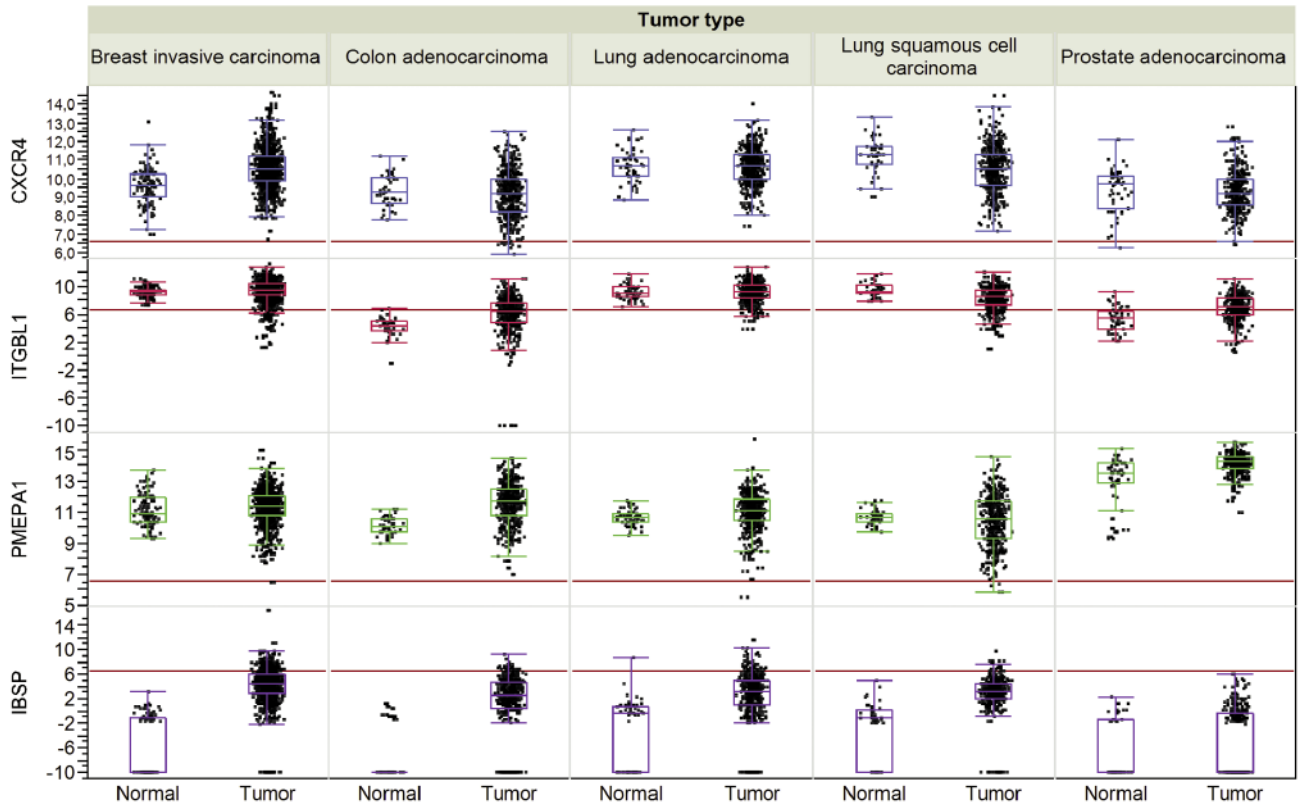
Figure 2. *RNA expression of selected bone metastasis-related genes in tumor and corresponding normal tissues. Cohorts from The Cancer Genome Atlas (TCGA) for invasive breast carcinoma (1,046 tumors, 111 normal tissues), colon adenocarcinoma (439 tumors, 41 normal tissues), lung adenocarcinomas (466 tumors, 58 normal tissues), lung squamous carcinoma (419 tumors, 45 normal tissues) and prostate adenocarcinoma (360 tumors, 51 normal tissues) were analyzed. Data for CX chemokine receptor 4 (CXCR4), integrin β-like 1 (ITGBL1), prostate transmembrane protein, androgen induced 1 (PMEPA1) and bone sialoprotein (BSP), are shown. Expression was measured by whole transcriptome sequencing and values provided represent normal read counts (log2), as derived from the Broad FIREHOSE portal. The red line represents a normalized read count of 100 reads and may help separate low from higher expression. Boxes contain 50% of the samples within the box while the whiskers include all other samples except for outliers. Normal tissue represents matched adjacent normal tissue of cancer patients.*

dentin sialo-phosphoprotein (DSPP) and matrix extracellular phosphoprotein (MEPE). Binding to integrin- αvβ3 and CD44 confers motility and survival signals to tumor cells. SIBLINGS are also involved in activation of MMPs and they prevent complement activation by binding to complement factor H (59). High levels of expression of SIBLINGS were detected in osteotropic cancers such as breast-, prostate- and lung cancer (59). A number of observations support their functional role as mediators of bone metastasis. A microarray and functional genomics-based study shows that OPN and BSP are functionally involved in bone metastasis of breast cancer cells (54). Antisense inhibition of OPN and BSP inhibits osteolytic metastasis of human breast cancer cells (60, 61). The role of host-derived OPN was underlined by the demonstration that melanoma cells, that do not express OPN showed reduced lung and bone metastasis when injected into OPN-deficient mice in comparison to wild-type mice (62). OPN can also promote tumor growth, block TNF-related apoptosis inducing ligand (TRAIL) and stimulate growth of endothelial cells (63). Furthermore, transfection of an expression construct for BSP into a brain-metastasizing breast cancer cell line was sufficient to induce bone metastasis (64). Shared gene expression profiles have been found between human breast cancer cell lines with a high propensity for metastasis to the bones and osteoclasts (59). Therefore, osteomimetic properties might favour the seeding of disseminated tumor cells in the skeleton by improving adhesion, proliferation and survival in the bone. The Cancer Genome Atlas (TCGA)-based analysis of RNA steady-state levels for BSP in breast, colon, lung and prostate carcinoma in comparison to their corresponding matching normal tissues revealed overexpression in tumor tissues, also in colon

carcinoma with no pronounced tropism of metastasis to the bones (Figure 2). However, the patients shown in our panel are not stratified with respect to risk or occurrence of metastasis.
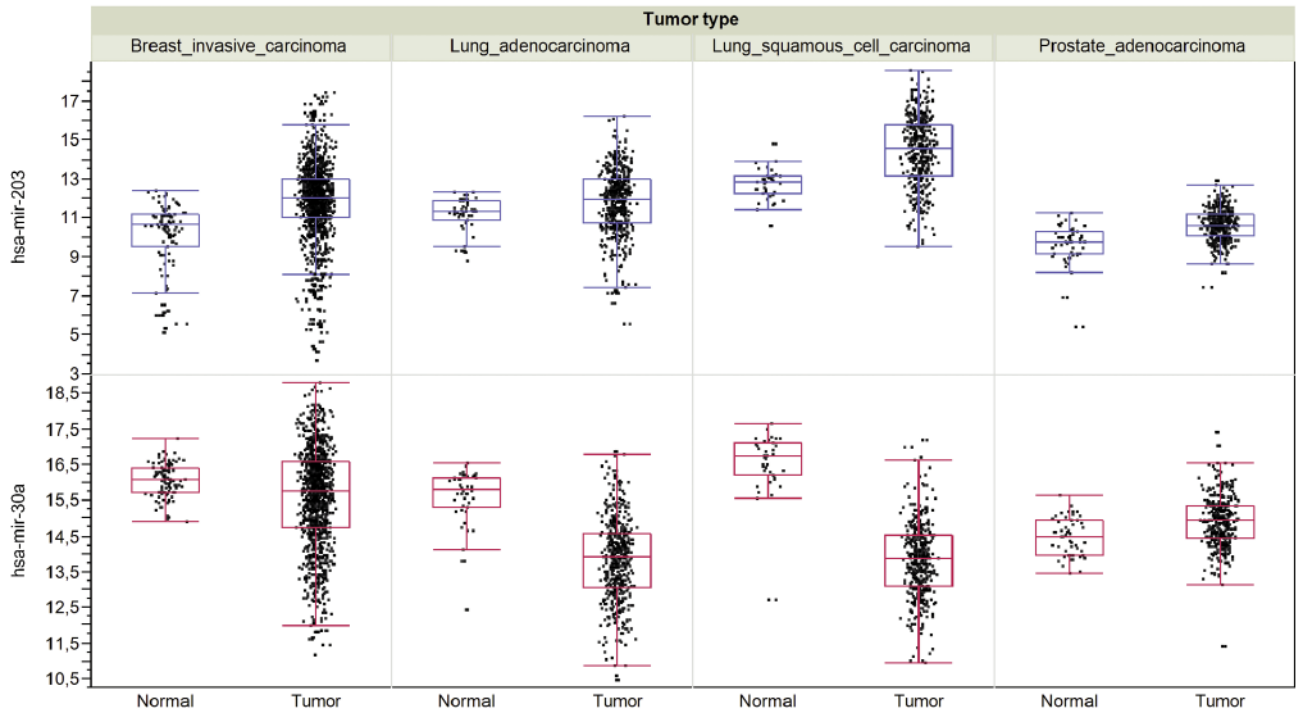
Identification of genes mediating bone metastasis. Genes mediating metastasis of breast cancer cells to the bones were identified with a model system based on MDA-MB-435 cells (54). Sub-clones with high tropism for bone metastasis were isolated by repeated *in vitro* culture and intra-cardiac injection of cells derived from bone metastases. The most highly overexpressed genes in the metastatic variants are *CXCR4*, involved in homing (65); matrix metalloproteinase 1, a mediator of invasion (66, 67); connective tissue growth factor (*CTGF*) and fibroblast growth factor 5 (*FGF5*) which are promoters of angiogenesis (68, 69); as well as osteolysis-promoting proteins IL11 (70) and OPN (59). *IL11* and *CTGF* are genes which are induced by canonical TGF-β signaling. Since TGF-β is released concomitantly with osteolysis, a positive feedback system is maintained in this model system. Expression of individual genes, as described above, in poorly-metastasizing breast cancer cells did not, or only modestly enhance metastasis to the bones. However, combination of IL11 and OPN dramatically increased bone metastasis and combinations of IL11 and OPN either with CXCR4 or CTGF gave rise to cell lines with aggressive bone metastasis comparable to the sub-clones selected for metastasis experiments. However, the signature of metastasis-promoting genes of this model system does not match with the 70-genes poor-prognosis signature for breast cancer (71). This may be due to the fact that only a fraction of breast cancers profiled to derive the signature, metastasize to the bones or that overexpression of the identified metastasis-promoting genes may occur late in the course of the disease. Such genes would not be scored in studies for bad prognosis signatures shared by a large proportion of primary breast cancers. Another possible explanation is that there was a selection of stochastically de-regulated factors rather than a selection for a set of specific genes. The genes, as described, are not contained in the MammaPrint (Agendia NV, Amsterdam, Netherlands) and the Oncotype DX (Genomic Health, Redwood City, CA, USA) commercial assays which are applied for prognosis in patients with early-stage breast cancer.

Breast cancers with high expression of integrin β-like 1 (ITGBL1) are prone to metastasize to the bone (72). ITGBL1 is highly homologous to the epidermal growth factor (EGF)-like stalk of β1 integrin and contains 10 EGF-like repeat domains, but no transmembrane or RGD (Arg-Gly-Arg) binding domains (73). ITGBL1 is overexpressed in bone metastatic sub-clone cells and is included in the osteoblast-like gene expression signature and bone metastastatic signature emphasizing its role in the osteomimetic phenotype

(72). ITGBL1 is a target of transcription factor Runx2 and induces expression of TGFβ1 and TGFβ3 as mediators of bone metastasis promoting function. ITGBL1 is a potential marker for prediction of metastatic risk of breast cancer and a potential therapeutic target. Steady-state levels of ITGBL1 RNA, as derived from TCGA were not increased in breast- and lung carcinomas in comparison to its normal tissue counterparts, however, in colon- and prostate carcinomas, the tumor tissues revealed overexpression of this gene (Figure 2). From this data set a correlation of expression of ITGLB1 RNA with bone tropism of metastasis cannot be deduced since breast- and lung carcinomas metastasize to the bones. However, the patients in our panel are not stratified with respect to risk or occurrence of metastasis.

Prostate transmembrane protein, androgen induced 1 (PMEPA1) was identified as another regulator of TGF-β signaling with metastasis-suppressing function of prostate cancer metastasis to the bones (74). PMEPA1 interferes with pro-metastatic TGF-β signaling by interaction with receptor-regulated SMADs (R-SMADs) and ubiquitin ligases (74). PMEPA1 was found to be decreased in metastatic prostate cancer patients and low PMEPA1 correlates with decreased metastasis-free survival (74). PMEPA1 is a potential marker for patient stratification with respect to risk of bone metastasis and a possible therapeutic target. However, RNA expression analysis, as derived from TCGA, did not reveal down-regulation of PMEPA1 expression in tumor tissues of breast, colon, lung and prostate carcinoma in comparison to matching normal tissue counterparts (Figure 2). One should keep in mind that data regarding risk or occurrence of metastasis for these patients are not available.

CXCL12/CXCR4 signaling axis and bone metastasis. The chemokine/chemokine receptor CXCL12/CXCR4 pathway has been shown to be involved in bone metastasis (75). Stromal cells secreting CXCL12 are able to attract CXCR4-overexpressing tumor cells (76). This pathway is primarily involved in mobilization of hematopoietic stem cells and the creation of a niche for cancer stem cells (76). Also, activation of CXCL12/CXCR4 signaling has been shown to be implicated in tumor cell proliferation and angiogenesis (76, 77). CXCL12/CXCR4 signaling was explored in the context of metastasis of prostate cancer cells to the skeleton. In an *in vivo* metastasis model, CXCR4 monoclonal antibodies (mAbs) and CXCR4 blocking peptides inhibited bone metastasis of prostate cancer cells as well as their growth after intratibial injection (78). A crucial finding was the demonstration that this signaling axis was involved in adhesion of prostate cancer cells to bone marrow endothelial cells (65, 78, 79). The role of CXCL12-induced integrin αvβ3 activation in adhesion of prostate cancer cells to endothelial cells is further supported by inhibition experiments with an αvβ3mAb (80, 81). However, adhesion

Figure 3. *Expression of selected bone metastasis-related miRNAs in tumor and corresponding normal tissues. Data for human miRNAs hsa-mir-203 and hsa-mir-30a and derived from The Cancer Genome Atlas (TCGA), are shown. TCGA cohorts for invasive breast carcinoma (1,046 tumors, 111 normal tissues), lung adenocarcinomas (466 tumors, 58 normal tissues), lung squamous carcinoma (419 tumors, 45 normal tissues) and prostate adenocarcinoma (360 tumors, 51 normal tissues) were analyzed. Expression was measured by short RNA sequencing and provided values represent normal read counts (log2), as derived from the Broad FIREHOSE portal. The red line represents a normalized read count of 100 reads and may help separate low from higher expression. Boxes contain 50% of the samples within the box while the whiskers include all other samples except for outliers. Normal tissue represents matched adjacent normal tissue of cancer patients.*

seems to be mediated by a sequence of interactions since an anti-CD164 mab can block binding of prostate cancer cells to endothelial cells. CD164 is overexpressed in prostate cancer metastases in comparison to primary tumors (82). The involvement of the CXCL12/CXCR4 axis in invasion is supported by induction of MMP9 and decrease in tissue-inhibitor of metalloproteinases 2 (TIMP2) expression in prostate cancer cells by this pathway (83, 84). In breast cancer, experimental evidence supports involvement of HER2/CXCR4/AKT signaling in bone metastasis (85). RNA steady-state levels for CXCR4 were clearly elevated in invasive breast carcinomas in comparison to their matching normal tissue counterparts in contrast to colon, lung and prostate carcinomas (Figure 2). These data may indicate a role of CXCR4 in the pathobiology of breast cancer. A role in bone metastasis of breast cancer cannot be deduced from these data, since the patients shown in our panel are not stratified with respect to risk or occurrence of metastasis.

miRNAs and their involvement in bone metastasis. miRNAs are small RNAs that regulate gene expression and can act as modulators of regulatory networks (86). They can trigger epithelial mesenchymal transition (EMT), mesenchymal epithelial transition (MET), osteomimicry as well as osteoblast and osteoclast function (87).

Regulation of EMT and MET by miRNA has been discussed in detail (50). miR203 was identified as a suppressor of bone metastasis (88). Low expression of miR203 correlates with prostate cancer progression and ectopic expression of miR203 in PC3 cells induces morphological changes from a fibroblast-like to an epithelial-like phenotype and decreases their metastatic propensity in a mouse model of bone metastasis (88). Based on TCGA data we found overexpression of miR203 in invasive breast carcinoma, adeno- and squamous cell lung carcinoma and prostate carcinoma in comparison to normal matching tissues (Figure 3). These data were derived from unstratified patients

and therefore a correlation to risk of metastasis cannot be deduced. Comparative analysis of primary tumors and matched metastases of breast cancer patients has shown that only metastatic cells express bone-related proteins such as osteonectin, cadherin 11, connexin 43, integrin αvβ3, cathepsin K and Runx2 (89, 90). miRNAs can also act as mediators of a transcriptional profile in tumor cells compatible with osteomimicry. miR218 is a pro-metastatic miRNA which up-regulates BSP, OPN and CXCR4 in breast cancer cells (91). The miR-30 family members are another class of mediators of osteomimicry. Comparative analysis of miRNAs in the breast cancer cell line MDA-MB 231 and its bone-metastatic variant MDA-B02 revealed down-regulation of the miR-30 family members in MBA-B02 cells (92). Restoration of expression of miR-30 family members in MDA-B02 cells decreased bone metastases (92). The clinical relevance of these findings is underlined by the fact that miR-30 family members have lower expression in lymph node metastases *versus* primary breast tumors (93). Low levels of miR33a correlate with cancer-mediated bone destruction (94). In lung cancer cells, restoring miR-33a expression decreases production of osteoclastogenesis activator RANKL and expression of PTHrP (94). RNA expression data derived from TCGA data reveal overexpression of miR-30a in non-stratified tumors of the prostate and down-regulation in patients with breast tumors as well as in adeno- and squamous cell lung carcinomas in comparison to matching normal tissues (Figure 3). A Phase I study for replacement therapy of miR-34, a suppressor miRNA that is commonly down-regulated in cancer, is ongoing in patients with hepatocellular carcinoma (95).

Treatment of bone metastasis. Bone metastases are associated with skeletal-related effects (SRE) such as hypercalcemia, bone pain, bone fractures and nerve compression leading to reduced mobility and quality of life. Bisphosphonates and Denosumab, a mAb directed against RANKL, are now established agents for the treatment of SRE and other agents are in clinical development (40, 96)

Bisphosphonates can be classified into nitrogen-free compounds such as clodronate and etidronate as well as nitrogen-containing compounds such as zoledronic acid, pamidronate, ibandronate and alendronate (97). Bisphosphonates inhibit binding of osteoclasts to bone and induce apoptosis of osteoclasts. Non-nitrogen-containing bisphosphonates are converted into methylene-containing analogs of ATP and accumulate in macrophages and osteoclasts, mediating apoptosis (98). Nitrogen-containing bisphosphonates inhibit farnesyl-diphosphate synthase, a rate-limiting enzyme of the mevalonate pathway, preventing prenylation of the GTPase activity of signaling proteins such as Ras, Rho and Rab in osteoclasts (99). Bisphosphonates

delay time of first SRE, but they do not prevent development of bone metastasis in patients with no bone metastases and they do not prolong survival (100). However, in an adjuvant setting, significant clinical benefit has been observed in a low estrogen microenvironment such as post-menopausal women or sub-groups with ovarian suppression (96). However, osteonecrosis of the jaw and nephrotoxicity have been observed as side-effects, whereas treatment-related hypocalcemia can be prevented by calcium and vitamin D supplementation (96).

Denosumab is a fully humanized immunoglobulin G2 (IgG2) mAb that prevents RANK/RANKL interaction (101). Denosumab has superior efficacy over zoledronic acid in preventing SRE (102). The effects of adjuvant denosumab on recurrence and survival are currently being investigated (96). N-telopeptide of type I collagens (NTX) has been identified as an excellent marker of bone resorption. The ratio between urinary NTX and creatinine is now routinely monitored as a measure for bone resorption (103).

Radionuclides (Sr-89, Rhe-186, Sm-153) are established agents for palliation of bone pain in patients with multifocal osteoblastic metastasis (40, 96). Dasatinib, an agent approved for the treatment of CML was shown to inhibit osteoclastogenesis (104) and sarcatinib, another src inhibitor decreased bone resorption markers in a Phase I study (105).

Cathepsin K, a lysosomal cysteine protease secreted from osteoclasts is also involved in osteolysis and a small molecule inhibitor, odanacatib, suppresses bone resorption similar to zoledronic acid (106). BHQ 880, a fully human mAb promoting osteoblastogenesis by inhibition of dickkopf-1 (DKK-1), an inhibitor of Wnt signaling, is presently evaluated in patients with multiple myeloma (107). In multiple myeloma, agents such as proteasome inhibitor bortezomib and immune-modulating drugs such as lenalidomide and pomalidomide inhibit osteoclastogenesis and are under further clinical investigation (108). Finally, agents antagonizing TGFβ and PTHrP are under clinical investigation in patients with bone metastases (109, 110).

Concluding Remarks

As outlined, crucial pathways involved in metastasis of tumors with bone tropism of metastasis have been identified. Progress in understanding the formation of pre-metastatic niches and the molecular basis of dormancy of disseminated tumor cells in the bone marrow are also based on recent knowledge. Clearly, more pre-clinical *in vivo* models mimicking specific clinically-relevant scenarios are crucial for the design of clinical approaches for prevention and treatment of bone metastases. Treatment of skeletal-related events with bisphosphonates and denosumab are impressive clinical achievements. However, it is not yet resolved, whether combination therapies with these agents in an adjuvant setting will result in survival benefit in

selected patient populations. The elucidation of tumor-type specific mechanisms for bone metastasis might result in targets for tumor-type specific prevention and treatment of bone metastases.

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