Contribution of Bone Tissue Modulus to Breast Cancer Metastasis to Bone

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Abstract Certain tumors, such as breast, frequently metastasize to bone where they can induce bone destruction. Currently, it is well-accepted that the tumor cells are influenced by other cells and growth factors present in the bone microenvironment that lead to tumor-induced bone disease. Over the past 20 years, many groups have studied this process and determined the major contributing factors; however, these results do not fully explain the changes in gene expression and cell behavior that occur when tumor cells metastasize to bone. More recently, groups studying

metastasis from soft tissue sites have determined that the rigidity of the microenvironment, which increases during tumor progression in soft tissue, can regulate tumor cell behavior and gene expression. Therefore, we began to investigate the role of the rigid bone extracellular matrix in the regulation of genes that stimulate tumor-induced bone disease. We found that the rigidity of bone specifically regulates parathyroid hormone-related protein (PTHrP) and Gli2 expression in a transforming growth factor β (TGF- β) and mechanotransduction-dependent mechanism. In this review, we summarize the mechanotransduction signaling pathway and how this influences TGF- β signaling and osteolytic gene expression.

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Introduction-The Vicious Cycle

Tumors such as breast, prostate, lung and others frequently metastasize to bone where they cause significant morbidity and mortality in patients. While current treatments with bisphosphonates have greatly reduced the number of skeletal related events experienced by patients, there are no effective treatments to inhibit tumor growth, and thus bone metastases remain incurable [1]. Therefore, there is a compelling clinical need for new drugs for both inhibiting tumor cell growth in bone as well as the resulting bone disease.

When tumor cells reach the bone they can cause bone destruction, bone formation, or a mixture of both. In the case of osteolytic disease, which is most common in breast cancer patients, tumor cells metastasize to bone where the abundance of growth factors in the bone microenvironment

can cause changes in gene expression that drive tumor cell production of osteolytic factors [2, 3]. While many groups have identified several relevant pathways that promote bone destruction, our work has focused on the production of PTHrP that stimulates osteoclast-mediated bone resorption and the release of TGF- β . This release of TGF- β from the bone matrix further stimulates PTHrP expression, causing a further stimulation of bone destruction.

It is now well understood that communication occurs not only between the tumor cells and bone, but also includes other cell types, such as immune cells and stromal cells. Additionally, the physical microenvironment of the bone may contribute to changes in tumor and bone cell behavior. Taking this into account helps explain the unique characteristics of tumor cell gene expression and behavior when tumor cells metastasize to bone.

The Bone Micro-Environment

Bone is a unique microenvironment that is different than any other tissue in the body. One of the major differences between bone and soft tissue is the rigidity of the mineralized extracellular matrix, which is approximately 1,000,000 times more rigid than normal breast tissue. The rigid bone matrix not only causes physical constraints on tumor growth, but it also alters both tumor cell gene expression and tumor cell response to growth factors [4]. While the rigid mineralized extracellular matrix is a major factor in the regulation of tumor cell gene expression, other characteristics of the bone microenvironment, such as stromal cells, may also mediate the behavior of the tumor cells when they become established in bone. Bone marrow stroma is a diverse population of cells that consists of precursor cells, immune cells, and fibroblasts. There is sufficient evidence from many different studies that each of these cell types significantly contributes to the overall gene expression and behavior of tumor cells that metastasize to bone [5].

Growth Factors

While bone is frequently considered to be an organ made of primarily structural proteins, it is also a vast storage site for many different growth factors, such as TGF- β , BMPs, Wnts, and Insulin-like growth factor (IGFs). As osteoclasts resorb the extracellular matrix, the release of these growth factors potentially affects tumor cell growth and behavior. Each of these factors contributes to physiological bone formation in different ways, and all are likely involved in tumor-induced bone disease; however, the role of TGF- β in this process has been studied most extensively. For

example, TGF- β released during bone destruction stimulates the expression of the transcription factor Gli2 leading to increased expression of PTHrP [6].

TGF- β has a complicated role both in the regulation of tumor growth and in bone formation. In the regulation of tumor growth, TGF- β is well-documented to play an important biphasic role, wherein it inhibits proliferation of normal cells and tumor cells in the early stages of growth. However, at later stages of growth, tumor cells, through transcriptional inhibition, stop expressing TGF- β receptor type II (TGF β RII), causing them to no longer respond to the growth inhibitory effects of TGF- β [7–9]. Interestingly, tumor cells that are known to induce bone destruction express TGF β RII, suggesting that the tumor cells may re-express the receptor once established in bone [10]. In bone metastases, TGF- β response is critical for the initiation and continuation of bone destruction. When tumor cells are genetically altered to no longer express TGF β RII, tumor bearing mice display a reduction in bone destruction [11]. This is in part through the ability of TGF- β to regulate the expression of the osteolytic factor, PTHrP, through the Smad and MAPK pathways [12]. In addition, TGF- β is known to be a critical component of the cellular response to tissue rigidity. This TGF- β mediated response to rigidity likely contributes in part to the biphasic and complicated regulation of TGF- β seen both in normal tissue and bone.

Tumor Produced Factors

Several tumor-produced factors have been studied by various groups through the years. Some of these include PTHrP, Runx2, interleukin-8 (IL-8), macrophage colony-stimulating factor (M-CSF), and others. The effects of PTHrP in cancer are perhaps the best described. Professor T.J. Martin's group discovered in the 1990s that PTHrP was preferentially expressed when tumor cells metastasized to bone rather than soft tissue sites [13, 14]. Dr. Gregory Mundy's group has since shown that PTHrP expression is an important component of tumor-induced bone disease and that inhibiting its expression reduces bone destruction [11, 15, 16]. This production of PTHrP stimulates the osteoblasts to produce RANKL, which stimulates osteoclast-mediated bone resorption [2, 5]. Similarly, Runx2, which is expressed by breast tumors, has been reported to stimulate the expression of RANKL by the tumor cells, which can lead to bone destruction [17, 18]. M-CSF has also been demonstrated to be expressed by breast and prostate cancer cells and stimulate bone destruction through RANKL. Other molecules that have been associated with bone resorption are the interleukins, specifically IL-8, which is expressed by some tumors cells and can stimulate bone destruction independently of RANKL [19, 20]. While other factors have been implicated in bone disease, there is strong pre-clinical

evidence that the inhibition of PTHrP expression can reduce bone destruction [6, 12, 16, 21]. Due to this strong evidence, clinical trials were undertaken with antibodies for PTHrP; however, the results of these trials were never published.

PTHrP was initially discovered as a factor associated with humoral hypercalcemia of malignancy [22], but also plays an important role developmentally. Specifically, PTHrP is critical for endochondral bone formation, where deletion of PTHrP leads to a short-limbed dwarfism phenotype [23]. It is also important during mammary development and lactation, and release during lactation is thought to induce bone resorption for the release of calcium from the bone [24, 25]. Interestingly, in non-bony tissues the expression of PTHrP in smooth muscle cells has been demonstrated to be regulated by mechanical stretch or shear flow, as first described in the early 1990s in bladder [26], aorta [27, 28], uterus [29], and stomach [28, 30] tissues. Similarly, several studies in osteoblasts and chondrocytes have indicated that mechanical loading can enhance PTHrP expression [31, 32]. Taken together, these studies show that stretching of the cells can induce PTHrP expression in a variety of cells and tissues.

In tumor cells, PTHrP is regulated by TGF- β [11, 12] and the Hedgehog transcription factor Gli2 [6, 15]. Most studies suggest that TGF- β is a primary regulator of PTHrP, but although TGF- β expression is higher in bone, it is also expressed in most other tissues in the body. Thus it is unclear whether the higher concentration of TGF- β in bone can account for the differential expression in tumor-produced PTHrP [33]. As shown in Fig. 1a, while mechanical stretch differs from cellular attachment to a rigid matrix, the mechanotransduction pathway is activated in both cases through the action of external forces on the cell. Thus, recent observations showing that Gli2 and PTHrP expression are upregulated on rigid matrices through both mechanically transduced and TGF- β signaling [4] are consistent with previous studies reporting increased PTHrP expression in response to mechanical stretch [31, 32]. Interestingly, PTHrP and Gli2 expression were increased on rigid (compared to compliant) 2D substrates, but the effect was blocked when TGF- β or mechanically transduced signaling was inhibited. A more detailed description of the proposed mechanism driving changes in TGF- β response due to matrix rigidity will be presented in the following sections, which may explain the increased expression of PTHrP when tumor cells become established in bone.

The Role of Matrix Rigidity in Regulating Cellular Outcomes

When tumor cells metastasize to bone, they are in contact with a mineralized extracellular matrix that is approximate-

ly 1,000,000 times more rigid than soft tissue. The bone extracellular matrix comprises several matrix proteins, including collagen, fibronectin (FN), osteonectin, osteopontin, and thrombospondin 2 [34], each of which contributes to the structure and properties of the bone microenvironment. Integrin $\alpha_v\beta_3$, which is a receptor for the bone matrix proteins osteopontin, FN, and vitronectin, has been identified as a critical integrin in breast cancer and prostate cancer skeletal metastasis [35–41]. Overexpression of $\alpha_v\beta_3$ in breast cancer cells has been associated with increased levels of bone metastasis, tumor burden, and subsequent osteolysis [36, 42–45]. Considering the role of FN in tumor-induced bone disease, in vitro studies investigating the role of matrix rigidity on osteolytic gene expression have utilized FN as the matrix protein supporting binding of the tumor cells to the matrix [4, 46]. Alternatively, tumor cells may bind to the lining cells directly in contact with the bone through the bone stromal ligand osteopontin [47], which is required for tumor cell colonization to bone [48]. It is unclear whether tumor cells directly contact the bone or whether they attach to cells lining the bone. Regardless of whether tumor cells are in direct or indirect (i.e., through stromal cells) contact with the bone, the forces experienced by tumor cells in the mineralized bone microenvironment are anticipated to be greater than in soft tissue.

Cells respond to the rigidity of the extracellular matrix through integrins clustered at adhesion sites, which provide a bond between the matrix and the cytoskeleton of the cell [49]. Thus the response of cells to the rigidity of the matrix is regulated by a variety of mechanisms, including the strength of the integrin-matrix bond, the pulling capacity on these bonds, and the sensitivity of the mechanotransduction system [50]. The rigidity of the extracellular matrix is known to regulate tumor cell invasiveness at the primary site [51–53], and a recent in vitro study suggests that the rigid mineralized bone matrix contributes to the initiation of osteolytic gene expression by tumor cells when they become established in bone [46]. In this study, Gli2 and PTHrP expression increased when breast cancer cells were cultured on 2D substrates with elastic moduli exceeding that of the basement membrane ($\sim 10,000$ nN/ μm^2) [4, 46]. Considering that all the substrates were coated with the same surface concentration of FN, differences in gene expression could not be attributed to differences in composition. Since ROCK [4] and $\alpha_v\beta_3$ integrin are required for the increased osteolytic gene expression on rigid matrices, in a later section we review the role of integrins and ROCK in regulating the ability of the cell to sense the rigidity of the matrix.

The effects of the rigid mineralized bone extracellular matrix on expression of other factors associated with invasion and metastasis are largely unknown. The most

Fig. 1 PTHrP expression is regulated by mechanotransduction. **a** Mechanical stretch of smooth muscle cells results in external forces being applied to the cells (*black arrows*), which the cells may resist by applying a larger contractile force (*blue arrows*). As shown in the Northern blot, PTHrP mRNA levels in gradually distended bladders increased over time compared to bladders that were kept empty (Lower figure from [26]). Rat bladders were emptied of residual urine by aspiration with a 27-gauge needle. A group of bladders ($n=2$) were kept empty by cutting the ureters, while a second group ($n=2$) were distended gradually by accumulation of urine. Bladders were recovered at the times indicated in the figure after treatment. Control

bladders (indicated as 0 h) were obtained immediately after anesthesia. Thus mechanical stretch increases PTHrP expression by smooth muscle cells in bladder tissue. **b** Similarly, MDA-MB-231 tumor cells cultured on rigid substrates generate larger contractile forces (*blue arrows*) than on compliant substrates [50, 59]. As shown in the plot, both mRNA expression (*red bars*) and protein secretion (*black bars*) of PTHrP increased with the elastic modulus of the 2D substrate on which they were cultured. MRNA expression was measured at 24h by RT-PCR using 18S as an internal control. PTHrP protein secretion was measured in conditioned medium at 48h using a two-site immunoradiometric assay (IRMA) and normalized for cell number

relevant investigations providing evidence of other pathways that may respond to matrix rigidity are mechanical loading studies, where factors such as Wnt, IGF [54], TGF- β , and BMP-2 [55] have been shown to be upregulated with increased mechanical loading. How expression of these factors changes with variations in matrix rigidity is unknown but is of great interest, since they have also been implicated in tumor-induced bone disease.

Measurement of Matrix Rigidity

The parameter frequently used to quantify the rigidity of tissues and synthetic matrices is the elastic (or Young's) modulus, which unlike compliance or stiffness is independent of the geometry of the material. As shown in Fig. 2, the elastic modulus is defined by the initial slope of the stress (σ) versus strain curve and has SI units of N/m² or Pascal (Pa). As recently suggested [50, 56], we will use the proposed units for stress of nN/ μ m² (equivalent to kPa) that are more natural for tissue. The elastic moduli of various tissues are listed in Table 1. It is important to note that tissues, as well as natural and synthetic matrices used to investigate the effects of rigidity on cell fate, exhibit viscoelastic mechanical properties, where the relationship

between stress and strain is not linear but dependent on the magnitude of the force and the time scale of the deformation [50]. However, for the typically small deformations (e.g., 100–150 nm [57, 58]) relevant to mechanotransduction, the elastic modulus is a reasonable approximation for the rigidity of the matrix. In response to an applied external force, cells generate contractile traction forces against the matrix that are mediated by Rho-associated kinase (ROCK). Recent studies have suggested that in order for cells to respond to matrix rigidity, a threshold force must be obtained that generates a matrix displacement of approximately 100–150 nm [50, 57, 58]. Thus when cultured on rigid substrates, cells generate larger traction forces than on compliant substrates, which may be due to the fact that rigid substrates require larger contractile forces to maintain matrix strain [50, 59].

Integrins Mediate Interactions Between the Cell and the Extracellular Matrix (ECM)

Integrins, a family of transmembrane receptors, are the primary mediator of adhesions between cells and the matrix [60], and their function in mediating cell adhesion has been reviewed extensively [50, 61]. The extracellular domain of

Fig. 2 Definitions of (a) the elastic modulus, E , and (b) stiffness, k . The stiffness is related to the modulus by the expression $k=AE/L$, where A is the cross-sectional area and L is the length of the sample. The modulus is a convenient parameter for describing the rigidity of a matrix because it is independent of the geometry of the test specimen

integrins binds to ligands in the matrix, such as fibronectin, vitronectin, or laminin [62, 63], while the cytoplasmic domains bind to proteins associated with cytoskeletal linkage and signaling [61, 64]. Integrin binding to the ligands in the matrix results in integrin clustering and activation of enzymes including kinases (e.g., focal adhesion kinase (FAK)) and phosphatases (e.g., tyrosine phosphatase- α (RPTP- α)) that regulate signal transduction within the cell [50, 61]. RPTP- α can activate Src family kinases (SFKs) within about 300 ms after application of a force on fibronectin beads [65–67]. Phosphorylation of mitogen-activated protein kinase (MAPK) has also been reported to increase in response to mechanical stress, suggesting that integrins sense forces regulating gene expression by activation of the MAPK pathway [68], which is known to be downstream of TGF- β signaling [69].

At locations where integrins cluster on 2D substrates, other molecules are subsequently recruited to form focal adhesions [61], which can result from the application of an external force (e.g., a cellular contractile force) to integrins [70–73]. On a rigid substrate, the contractile force exerted

by the cells is greater than the force generated on a compliant substrate, resulting in increased clustering of integrins [51] and reinforcement of the bonds between the integrins and cytoskeleton [74]. Molecules such as RPTP- α [73], vinculin [71], and talin [72] strengthen the links between integrins and the cytoskeleton in a force-dependent manner. Forces applied to the cell also activate the RhoA/ROCK-mediated assembly of actomyosin stress fibers that exert contractile force and generate cytoskeletal tension [75]. Thus cells cultured on rigid substrates show up-regulated integrin expression [76], more stress fibers [76], stable focal adhesions [59, 71], and greater traction forces [51]. Conversely, cells cultured on compliant substrates show dynamic adhesions [59] and are not as spread [76].

Materials for Investigating Mechanotransduction Pathways

Many studies investigating the effects of the rigidity of the matrix on cell migration, differentiation, and invasion have used 2D cell culture on hydrogel substrates, including Matrigel™ [77], crosslinked gelatin [78], and synthetic hydrogels [79]. Similarly, the invasiveness of cancer cells has been reported to increase with matrix rigidity when cultured on hydrogels [51, 52, 78, 80]. However, considering that the elastic modulus of hydrogel substrates is generally $<100 \text{ nN}/\mu\text{m}^2$, their applicability to the bone microenvironment, where the modulus of the mineralized extracellular matrix ranges from 17,100,000–28,900,000 $\text{nN}/\mu\text{m}^2$ [50], may be limited.

Recent studies have investigated the effects of matrix rigidity on cellular outcomes over a range of elastic moduli that is more representative of the bone microenvironment. Both co-polymer chemistry and substrate rigidity were observed to regulate osteoblast differentiation of MG63 pre-osteoblast cells cultured on 2D acrylate-based co-polymer networks with elastic moduli ranging from 5,500–851,000 $\text{nN}/\mu\text{m}^2$ [81]. Another recent study has reported

Table 1 Rigidity of selected tissues. Table adapted from Butcher et al. 2009 [83] and Moore et al. 2010 [128]

Tissue Type	Elastic Modulus $\text{nN}/\mu\text{m}^2$	Synthetic substrates with elastic moduli in the same range
Brain	0.1–10	Polyacrylamide (PAA) hydrogels (48), matrigel, collagen (41), gelatin (67)
Lung	0.4	PAA hydrogels (48), matrigel, collagen (41), gelatin (67)
Breast	0.8	PAA hydrogels (48), matrigel, collagen (41), gelatin (67)
Muscle	12–100	PAA(123) and poly(ethylene glycol) PEG(124) hydrogels
Fat	20	PAA and PEG hydrogels, gelatin
Artery	100–3,800	Polyurethane (PUR) (2, 123) and poly(ethylene glycol) dimethacrylate (PEGDMA)/ diethyleneglycol dimethacrylate (DEGDMA) (70) networks
Areolar connective tissue	60–100	PAA (123) and poly(ethylene glycol) PEG (124) hydrogels
Smooth muscle	5,000	PUR (2, 123) and PEGDMA/DEGDMA (70) networks
Bone	17,100,000–28,900,000	PUR networks (2, 123), tissue culture polystyrene, glass, dentin

that expression of the osteogenic transcription factor RUNX2 increased on rigid 2D substrates [82]. MC3T3 pre-osteoblast cells were cultured on a 14 nN/ μm^2 hydrogel, a 424 nN/ μm^2 hydrogel, or tissue culture polystyrene ($\sim 2,000,000$ nN/ μm^2). Inhibition of ROCK or the MAPK pathway decreased RUNX2 activity and delayed the expression of markers of osteoblast differentiation. To test the effects of substrate rigidity on osteolytic gene expression in tumor cells, we designed a 2D cell culture system using substrates with tunable elastic moduli [4]. Poly (acrylamide) (PAA) hydrogels were investigated as a model for breast tissue (0.8 nN/ μm^2) [83], poly(ester urethane) (PUR) films [84] as a model for tissues ranging from smooth muscle (5,000 nN/ μm^2) to trabecular bone (8,700,000–11,400,000 nN/ μm^2) [85], and glass (67,000,000 nN/ μm^2) as a model for cortical bone (17,100,000–28,900,000 nN/ μm^2) [50]. Gli2 and PTHrP expression were lowest on PAA hydrogels and increased with modulus for values up to 1,000,000 nN/ μm^2 , at which point it became constant (Fig. 1b). However, many other genes associated with metastasis [86] to bone showed minimal changes in expression [4]. Interestingly, matrix rigidity regulated osteolytic gene expression for modulus values ranging from 1,000–1,000,000 nN/ μm^2 , which exceeds that of areolar connective tissue and smooth muscle and approaches that of trabecular bone [4].

Maximal Force Generation in Cells

Recent evidence suggests that the ability of the cell to sense substrate rigidity involves uniform displacement of the substrate [50]. Several studies suggest that cells sense rigidity based on whether a threshold force is obtained when the displacement of the substrate is in the range of 100–150 nm [50, 57, 58]. The magnitude of the elastic modulus of the substrate that would likely result in a displacement of 100 nm has been estimated to be 10 nN/ μm^2 [50]. A mechano-chemical coupling model has also been developed to physically describe cell adhesion and spreading mediated by the rigidity of the substrate based on the classical Bell-Dembo theory that cell adhesion is regulated by reversible receptor–ligand interactions [87, 88]. The model predicts that the force generated by the cells increases dramatically when cultured on substrates ranging from 1–100 nN/ μm^2 , and that the adhesion strength equals $\sim 90\%$ of the maximum value at 100 nN/ μm^2 . Taken together, these studies suggest that cells are likely to be more responsive to the matrix when the rigidity is in the range of 1–100 nN/ μm^2 , which is in agreement with a recent review reporting that actomyosin appeared diffuse for cells cultured on soft gels, while cells cultured on rigid (e.g., 30–100 nN/ μm^2) gels or glass (67,000,000 nN/ μm^2) showed stress fibers and strong focal adhesions [59]. Based

on these observations, it has been suggested that cells cultured on substrates having a modulus equal to or greater than that of rigid gels (~ 100 nN/ μm^2) are in a state of isometric contraction [59]. It is interesting to note that this range is at least 1–2 orders of magnitude lower than the range of moduli over which osteolytic gene expression increases in MDA-MB-231 and RWGT2 tumor cells that metastasize to bone.

The Role of the Rigid Extracellular Matrix in Promoting Osteolytic Gene Expression

We have recently suggested that the rigid mineralized bone matrix, which is at least 1,000,000 times stiffer than soft tissue, plays a significant role in the initiation of osteolytic gene expression by tumor cells after they become established in bone [4, 46]. When MDA-MB-231 or RWGT2 tumor cells were cultured on rigid 2D substrates with moduli approaching those of mineralized bone tissue, PTHrP and Gli2 gene expression increased by 2–4-fold compared to culture on compliant substrates approximating soft tissue. This increase in gene expression on rigid substrates was mediated by Rho kinase (ROCK) and TGF β RII, but the details of the signaling mechanism remained unclear.

Recent studies have described the relationship between mechanically transduced and oncogenic signaling [51, 53]. Integrins function as mechanoreceptors [70] and mediate mechanotransduction by transferring forces to focal adhesions connecting integrins to the cytoskeleton [89]. Changes in the rigidity of the matrix alter the force balance in the cell, thereby creating a positive feed-back loop that can drive cell proliferation [53]. Cellular traction forces increase with matrix rigidity, resulting in activation of integrins and formation of focal adhesions, as well as stimulation of Rho/ROCK signaling and subsequent phosphorylation of myosin light chain (MLC), driving increased cell contractility, which feeds back to further increase the rigidity of the matrix. Interestingly, breast cancer cells cultured on relatively rigid gels showed increased expression of the undifferentiated malignant phenotype as well as increased Rho activity [51]. Furthermore, over-expression of constitutively active RhoV14 in normal mammary epithelial cells cultured on compliant matrices induced the malignant properties observed when the cells were cultured on rigid matrices. Similarly, blocking cell contractility via pharmacological inhibition of myosin II or ROCK abrogated the malignant phenotype observed on rigid gels. These observations suggest that the effect of matrix rigidity on cell transformation is driven by ROCK-generated contractility. However, inhibition of Erk was also observed to abrogate the malignant phenotype observed for cells over-expressing

constitutively active RhoV14, as well as to reduce ROCK-generated contractility, which supports the notion that cross-talk between the mechanotransducing Rho/ROCK and mitogenic EGFR/Erk pathways sustains a positive feed-back loop that may drive cell transformation.

As shown in Fig. 3, our recent findings point to similar solid-state and soluble growth factor signaling pathways driving expression of osteolytic factors by tumor cells when they initially become established in the bone microenvironment. As discussed in Section “The Bone Micro-Environment”, active $\alpha_v\beta_3$ integrin in breast tumor cells binds to FN [90], thereby enabling cancer cells to adhere to and interact with bone matrix to control early events in bone metastasis [45]. Furthermore, $\alpha_v\beta_3$ integrin is required by osteoclasts to resorb the mineralized bone matrix, and antagonists of $\alpha_v\beta_3$ integrins have been successfully used to inhibit osteoclasts and reduce bone

loss in models of osteoporosis and metastatic bone disease [45, 91]. Thus blocking $\alpha_v\beta_3$ has the potential to block osteolysis in addition to inhibiting the stimulatory effect of TGF β on PTHrP expression.

Crosstalk between integrins and growth factor receptors also regulates tumor growth and invasion [90]. Integrin $\alpha_v\beta_3$ interacts physically with the TGF β RII, resulting in TGF- β -mediated stimulation of MAPKs and Smad2/3-mediated gene transcription in mammary epithelial cells (MECs) during epithelial-mesenchymal transition (EMT) [92]. Consequently, we hypothesized that the rigid bone matrix drives changes in ROCK-generated contractility and integrin-dependent signaling that are distinct from the primary site, and that these changes specific to bone may alter TGF- β signaling within metastatic tumor cells to initiate expression of factors required for osteolysis. In our preliminary studies we have found that MDA-MB-231 cells

Fig. 3 Proposed solid-state and soluble signaling pathways regulating tumor cell behavior in bone (including clinical targets). (A) In the bone microenvironment, the rigid mineralized bone matrix drives increased osteolytic gene expression by tumor cells when they initially become established in the bone microenvironment. Cells respond to mechanical tension through the activation of integrins, Rho-GTPases, and SFKs. (B) The rigid matrix alters TGF- β signaling by promoting increased clustering of $\alpha_v\beta_3$ and TGF β RII, followed by Src phosphorylation of TGF β RII to activate MAPK signaling [92], an important upstream effector of PTHrP [69]. Thus cross-talk between mechanosensing ROCK and soluble TGF- β signaling pathways drives expression of Gli2 and PTHrP

cultured on 2D substrates approximating the rigidity of bone showed an increase in mRNA expression of β_3 integrin subunit and increased phosphorylation of MLC [4] compared to cells grown on a compliant substrate. Data from other groups has suggested an interaction between TGF β RII and the β_3 integrin subunit [92], and our unpublished data suggest that this integrin-receptor interaction increases on rigid matrices. These observations suggest that mechanotransduction alters TGF- β signaling within osteolytic tumor cells, as shown in the solid-state signaling box in Fig. 3a.

Integrins are essential for translating the rigidity of the microenvironment into intracellular signaling pathways. Cells respond to mechanical tension through the activation of integrins, Rho-GTPases, and SFKs [93]. In soft tissues, cells respond to mechanical stimulus by activating SFKs [66, 94, 95]. Thus changes in both the expression and clustering of integrins, as well as integrin-mediated signaling pathways induced by the rigid mineralized bone matrix, can activate SFKs, which might be essential for the ability of the cell to sense the rigidity of the matrix [96]. Specific inhibitors to Src tyrosine kinases, which are required to regulate integrin-cytoskeleton interactions, have shown promise in preclinical studies, reducing both tumor burden and incidence in bone [97, 98] as well as metastasis to bone [99]. After activation, SFKs promote coupling of the cytoskeleton to integrins through talin [100–102], which enables mechanical forces on the integrins to engage the integrin/matrix catch bond [50]. While the detailed mechanisms governing translation of mechanical forces sensed within cell-matrix adhesions to biochemical changes are not completely known, changes in the conformation of integrins in response to a rigid matrix are thought to result in tension-dependent changes in conformation of integrin-associated proteins, such as SFK, p130Cas and vinculin [96].

In addition to their role in mediating mechanotransduction, SFKs are also known to regulate TGF- β signaling [50]. Src phosphorylation of TGF β RII in response to TGF β RII and $\alpha_v\beta_3$ clustering has been implicated in EMT and is known to activate MAPK signaling [103], an important upstream effector of PTHrP [69]. Our preliminary data indicate that Src and $\alpha_v\beta_3$ are required for the stimulation of PTHrP and Gli2 through ROCK [4]. Similarly, the increased association of β_3 integrin subunit with TGF β RII on rigid substrates suggests that the rigid mineralized bone matrix activates MAPK signaling in MDA-MB-231 cells [103]. Taken together, these data suggest that cross-talk between mechanosensing ROCK and soluble TGF- β signaling pathways drives expression of Gli2 and PTHrP, as summarized in Fig. 3b. This mechanism is similar to that proposed for tumor cell invasion and proliferation mediated by cross-talk between Rho/ROCK

and EGFR/Erk pathways in the primary site [51, 53], but there is a significant difference. In soft tissue, forces resulting from ROCK-generated contractility can drive cytoskeletal remodeling, resulting in stiffening of the matrix surrounding the tumor [52, 83] that alters integrin-dependent signaling within the tumor cells [52, 104, 105]. This positive feedback loop driving matrix remodeling in the primary site can lead to a 100-fold increase in matrix rigidity [51]. In contrast, in the bone microenvironment tumor cells interact with a rigid matrix that is at least 100,000,000-fold stiffer than the primary site. Consequently, the rigid bone matrix is anticipated to drive changes in ROCK-generated contractility and integrin-dependent signaling immediately upon establishment of the tumor cells in the bone microenvironment.

Clinical Perspectives

A variety of therapies have been approved or are under clinical investigation for the treatment of metastatic bone disease, as summarized in Fig. 3. Bisphosphonates are currently the clinical standard of care for treating patients with bone metastases, and have been demonstrated in clinical trials to be safe and efficacious by reducing the risk of osteoporotic fractures [106]. While they have been tremendously successful in the treatment of osteoporosis and tumor-induced bone disease, they do not directly target the tumor cells, which are the root cause of the problem. Furthermore, some patients fail bisphosphonate treatment, and the recent discovery of clinical complications observed with long term bisphosphonate treatments, including osteonecrosis of the jaw and atypical subtrochanteric fractures, has made these drugs less ideal clinically [1, 107, 108]. The emerging drug for clinical use, Denosumab or XGEVA (Amgen), which targets RANKL, has been demonstrated to reduce bone turnover and increase bone density in clinical studies [109]. Interestingly, this drug seems to have the capability of inhibiting tumor growth, yet this seems likely to be an indirect effect [110]. However, XGEVATM also presents safety risks, including hypocalcemia and osteonecrosis of the jaw, though the prevalence is unclear due to limited clinical data [1].

Inhibition of TGF- β has been another active area of recent pre-clinical studies, which have thus far been very successful, with several groups demonstrating that inhibition of TGF- β reduces metastasis to soft tissues [111, 112] and bone [113]. In addition to direct effects on the tumor, treatment of healthy (i.e., tumor-free) mice with the TGF- β inhibitors 1D11 (Genzyme, Cambridge, MA) and SD-208 (Scios, Inc., Sunnyvale, CA) increases bone density and biomechanical properties as well as numerous measures of bone quality (e.g., trabecular bone architecture and mineral:

collagen ratio) [114, 115]. Blocking TGF- β also increased the numbers of osteoblasts and decreased the numbers of osteoclasts in the bone marrow of healthy mice. These data suggest that a TGF- β inhibitor may be an ideal drug for bone metastases, since it can both inhibit tumor growth and improve bone quality. However, their use may be complicated in patients due to the biphasic effects of TGF- β in tumor growth and its effects throughout the body on normal cells [116]. Specifically, in genetic mouse models in which TGF- β signaling is stimulated, TGF- β inhibits formation of mammary tumors at early stages, but can promote metastasis of tumors at later stages. These observations suggest that while TGF- β inhibitors may block bone resorption in patients with bone metastases, they could possibly increase tumor cell growth in the primary site. This would be a particularly important issue for patients that still have a primary tumor or have multiple metastases. Therefore, when considering these drugs for clinical use the effects on other cell types or tumors need to be considered thoroughly.

The limitations of all these drugs underscore the need for more specific inhibitors of tumor growth in bone. Inhibiting downstream molecules, such as mechanotransduction inhibitors or Gli2, could potentially be an alternative treatment strategy. The ROCK inhibitors Fasudil (Tocris Bioscience, Ellisville, MO) and Y-27632 have been used successfully in preclinical models of pulmonary and cardiac disease. Furthermore, clinical studies have shown that Fasudil is a safe and efficacious treatment for patients with severe pulmonary hypertension [117]. Fasudil is currently being used clinically for the treatment of cerebral vasospasms, and is also being evaluated in clinical studies for other cardiovascular uses, indicating that the drug is well tolerated by patients [118]. Considering that ROCK is required for Gli2 and PTHrP expression, as well as the clinical safety and efficacy of Fasudil in cardiovascular applications, ROCK inhibitors such as Fasudil may be a potential alternative strategy for treatment of tumor-induced bone disease.

Additionally, based on our current data, inhibition of the mechanotransduction pathway makes an attractive target. Potential targets include pathways with well-established inhibitors such as Src and Integrins. Similar to TGF- β , these drugs have both been tested in normal mice and been shown to inhibit osteoclast activity [41, 119, 120]. Our data suggest that their inhibition may also prevent PTHrP and tumor growth, therefore suggesting that these drugs will target both the tumor and the bone microenvironment. Consistent with other studies implicating $\alpha_v\beta_3$ integrin in tumor progression [121], our unpublished data suggest that $\alpha_v\beta_3$ integrin is the prevalent integrin complex involved in tumor response to matrix rigidity [122]. Therefore, monoclonal antibodies have been developed to inhibit integrin

activity [121], such as LM-609, which has been shown to inhibit tumor growth in pre-clinical studies [123]. Etaracizumab (the humanized form of LM609) is currently in clinical trials for metastatic melanoma, where it has been used successfully [124]. Other drugs in clinical trials include CNTO 95 (intetumumab) for treatment of melanoma and angiosarcoma [125], as well as cilengitide for the treatment of Glioblastomas [126].

Alternatively, we are highly optimistic that Gli antagonists (GANT) will provide a more specific approach for targeting the tumor and subsequent bone disease. The GANT drugs have been used in pre-clinical trials in many different tumor types, and have been very effective at reducing tumor burden [127]. Our recently published data demonstrate that Gli2 inhibition nearly eliminates tumor growth in bone, while reducing PTHrP expression in tumors that are present, which results in a reduction in tumor-induced bone disease [6]. Therefore, in addition to inhibiting tumor growth, they are anticipated to block both the mechanically transduced and TGF- β mediated expression of PTHrP. Furthermore, considering the limited expression of Gli proteins in adults, effects on normal tissues should be minimal.

In the last several years, many potential targets for inhibiting tumor-induced bone disease have been studied. Each of these compounds have their benefits and drawbacks. It is likely that a combination of multiple drugs will likely be the best approach. Which of these targets will succeed is difficult to determine from the current pre-clinical data, but we anticipate that a drug combination targeting both the micro-environment and the tumor growth will be most effective.

Conclusions

Over the past 20 years our view of the “vicious cycle” has adapted to include many different growth factors and cell types. Recent studies have indicated that the rigidity of the microenvironment can control tumor cell gene expression and behavior. While previous studies have been limited to osteolytic tumor cells, the rigid bone microenvironment is anticipated to alter gene expression of osteoblastic tumor cells as well. Future work in this area is needed to better understand the pathways involved in the response of tumor cells to rigidity, which will facilitate the development of improved clinical targets that not only inhibit tumor-induced bone destruction, but that can also kill the tumor cells residing in bone or prevent tumors from establishing in bone. Further investigation of mechanotransduction pathways are expected to reveal new interactions that will continue to expand our understanding of the vicious cycle.

References

1. Fizazi K, Carducci M, Smith M, Damiao R, Brown J, Karsh L, Milecki P, Shore N, Rader M, Wang H, Jiang Q, Tadros S, Dansey R, Goessl C (2011) Denosumab versus zoledronic acid for treatment of bone metastases in men with castration-resistant prostate cancer: a randomised, double-blind study. *Lancet* 377:813–822
2. Guise TA, Mundy GR (1998) Cancer and bone. *Endocr Rev* 19:18–54
3. Yoneda T, Sasaki A, Mundy GR (1994) Osteolytic bone metastasis in breast cancer. *Breast Canc Res Treat* 32:73–84
4. Ruppender NS, Merkel AR, Martin TJ, Mundy GR, Sterling JA, Guelcher SA (2010) Matrix rigidity induces osteolytic gene expression of metastatic breast cancer cells. *PLoS One* 5:e15451
5. Sterling JA, Edwards JR, Martin TJ, Mundy GR (2011) Advances in the biology of bone metastasis: how the skeleton affects tumor behavior. *Bone* 48:6–15
6. Johnson RW, Nguyen MP, Padalecki SS, Grubbs BG, Merkel AR, Oyajobi BO, Matrisian LM, Mundy GR, Sterling JA (2011) Tgf- β promotion of gli2 induced pthrp expression is independent of canonical hedgehog signaling. *Cancer Res*
7. Sterling JA, Wu L, Banerji SS (2006) Parp regulates tgf-beta receptor type ii expression in estrogen receptor-positive breast cancer cell lines. *Anticancer Res* 26:1893–1901
8. Ko Y, Koli KM, Banerji SS, Li W, Zborowska E, Willson JK, Brattain MG, Arteaga CL (1998) A kinase-defective transforming growth factor-beta receptor type ii is a dominant-negative regulator for human breast carcinoma mcf-7 cells. *Int J Oncol* 12:87–94
9. Ko Y, Banerji SS, Liu Y, Li W, Liang J, Soule HD, Pauley RJ, Willson JK, Zborowska E, Brattain MG (1998) Expression of transforming growth factor-beta receptor type ii and tumorigenicity in human breast adenocarcinoma mcf-7 cells. *J Cell Physiol* 176:424–434
10. Guise TA (2000) Molecular mechanisms of osteolytic bone metastases. *Cancer* 88:2892–2898
11. Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R, Massague J, Mundy GR, Guise TA (1999) Tgf-beta signaling blockade inhibits pthrp secretion by breast cancer cells and bone metastases development. *J Clin Invest* 103:197–206
12. Kakonen SM, Selander KS, Chirgwin JM, Yin JJ, Burns S, Rankin WA, Grubbs BG, Dallas M, Cui Y, Guise TA (2002) Transforming growth factor-beta stimulates parathyroid hormone-related protein and osteolytic metastases via smad and mitogen-activated protein kinase signaling pathways. *J Biol Chem* 277:24571–24578
13. Powell GJ, Southby J, Danks JA, Stillwell RG, Hayman JA, Henderson MA, Bennett RC, Martin TJ (1991) Localization of parathyroid hormone-related protein in breast cancer metastases: increased incidence in bone compared with other sites. *Cancer Res* 51:3059061
14. Southby J, Kissin MW, Danks JA, Hayman JA, Moseley JM, Henderson MA, Bennett RC, Martin TJ (1990) Immunohistochemical localization of parathyroid hormone-related protein in human breast cancer. *Cancer Res* 50:7710–7716
15. Sterling JA, Oyajobi BA (2006) The hedgehog signaling molecule gli2 induces parathyroid hormone-related peptide expression and osteolysis in metastatic human breast cancer cells. *Cancer Res* 66:7548–7553
16. Guise T, Yin J, Taylor S, Kumagai Y, Dallas M, Boyce B, Yoneda T, Mundy G (1996) Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. *J Clin Invest* 98:1544–1549
17. Pratap J, Javed A, Languino LR, van Wijnen AJ, Stein JL, Stein GS, Lian JB (2005) The runx2 osteogenic transcription factor regulates matrix metalloproteinase 9 in bone metastatic cancer cells and controls cell invasion. *Mol Cell Biol* 25:8581–8591
18. Javed A, Barnes GL, Pratap J, Antkowiak T, Gerstenfeld LC, van Wijnen AJ, Stein JL, Lian JB, Stein GS (2005) Impaired intranuclear trafficking of runx2 (aml3/cbfa1) transcription factors in breast cancer cells inhibits osteolysis in vivo. *Proc Natl Acad Sci USA* 102:1454–1459
19. Bendre MS, Margulies AG (2005) Tumor-derived interleukin-8 stimulates osteolysis independent of the receptor activator of nuclear factor-kappaB ligand pathway. *Cancer Res* 65:11001–11009
20. Bendre MS, Montague DC, Peery T, Akel NS, Gaddy D, Suva LJ (2003) Interleukin-8 stimulation of osteoclastogenesis and bone resorption is a mechanism for the increased osteolysis of metastatic bone disease. *Bone* 33:28–37
21. Gallwitz WE, Guise TA (2002) Guanosine nucleotides inhibit different syndromes of pthrp excess caused by human cancers in vivo. *J Clin Invest* 110:1559–1572
22. Broadus AE, Mangin M, Ikeda K, Insogna KL, Weir EC, Burtis WJ, Stewart AF (1988) Humoral hypercalcemia of cancer. Identification of a novel parathyroid hormone-like peptide. *N Engl J Med* 319:556–563
23. Lanske B, Amling M, Neff L, Guiducci J, Baron R, Kronenberg HM (1999) Ablation of the pthrp gene or the pth/pthrp receptor gene leads to distinct abnormalities in bone development. *J Clin Invest* 104:399–407
24. Wysolmerski JJ, Philbrick WM, Dunbar ME, Lanske B, Kronenberg H, Broadus AE (1998) Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. *Development* 125:1285–1294
25. VanHouten JN, Wysolmerski JJ (2003) Low estrogen and high parathyroid hormone-related peptide levels contribute to accelerated bone resorption and bone loss in lactating mice. *Endocrinology* 144:5521–5529
26. Yamamoto M, Harm SC, Grasser WA, Thiede MA (1992) Parathyroid hormone-related protein in the rat urinary bladder: a smooth muscle relaxant produced locally in response to mechanical stretch. *Proc Natl Acad Sci USA* 89:5326–5330
27. Noda M, Katoh T, Takuwa N, Kumada M, Kurokawa K, Takuwa Y (1994) Synergistic stimulation of parathyroid hormone-related peptide gene expression by mechanical stretch and angiotensin ii in rat aortic smooth muscle cells. *J Biol Chem* 269:17911–17917
28. Pirola CJ, Wang HM, Strgacich MI, Kamyar A, Cercek B, Forrester JS, Clemens TL, Fagin JA (1994) Mechanical stimuli induce vascular parathyroid hormone-related protein gene expression in vivo and in vitro. *Endocrinology* 134:2230–2236
29. Thiede MA, Daifotis AG, Weir EC, Brines ML, Burtis WJ, Ikeda K, Dreyer BE, Garfield RE, Broadus AE (1990) Intrauterine occupancy controls expression of the parathyroid hormone-related peptide gene in preterm rat myometrium. *Proc Natl Acad Sci USA* 87:6969–6973
30. Ito M, Ohtsuru A, Enomoto H, Ozeki S, Nakashima M, Nakayama T, Shichijo K, Sekine I, Yamashita S (1994) Expression of parathyroid hormone-related peptide in relation to perturbations of gastric motility in the rat. *Endocrinology* 134:1936–1942
31. Tanaka N, Ohno S, Honda K, Tanimoto K, Doi T, Ohno-Nakahara M, Tafolla E, Kapila S, Tanne K (2005) Cyclic mechanical strain regulates the pthrp expression in cultured chondrocytes via activation of the ca²⁺ channel. *J Dent Res* 84:64–68
32. Chen X, Macica CM, Ng KW, Broadus AE (2005) Stretch-induced pth-related protein gene expression in osteoblasts. *J Bone Miner Res* 20:1454–1461

33. Dallas SL, Miyazono K, Skerry TM, Mundy GR, Bonewald LF (1995) Dual role for the latent transforming growth factor-beta binding protein in storage of latent tgf-beta in the extracellular matrix and as a structural matrix protein. *J Cell Biol* 131:539–549
34. Young MF (2003) Bone matrix proteins: their function, regulation, and relationship to osteoporosis. *Osteoporos Int* 14(Suppl 3):S35–S42
35. Yoneda T (2000) Cellular and molecular basis of preferential metastasis of breast cancer to bone. *J Orthop Sci* 5:75–81
36. van der P, Vloedgraven H, Papapoulos S, Lowick C, Grzesik W, Kerr J, Robey PG (1997) Attachment characteristics and involvement of integrins in adhesion of breast cancer cell lines to extracellular bone matrix components. *Lab Invest* 77:665–675
37. Liapis H, Flath A, Kitazawa S (1996) Integrin alpha v beta 3 expression by bone-residing breast cancer metastases. *Diagn Mol Pathol* 5:127–135
38. McCabe NP, De S, Vasanji A, Brainard J, Byzova TV (2007) Prostate cancer specific integrin alphavbeta3 modulates bone metastatic growth and tissue remodeling. *Oncogene* 26:6238–6243
39. Townsend PA, Villanova I, Uhlmann E, Peyman A, Knolle J, Baron R, Teti A, Horton MA (2000) An antisense oligonucleotide targeting the alphav integrin gene inhibits adhesion and induces apoptosis in breast cancer cells. *Eur J Cancer* 36:397–409
40. Gillespie MT, Thomas RJ, Pu ZY, Zhou H, Martin TJ, Findlay DM (1997) Calcitonin receptors, bone sialoprotein and osteopontin are expressed in primary breast cancers. *Int J Canc* 73:812–815
41. Schneider JG, Amend SR, Weillbaeher KN (2011) Integrins and bone metastasis: integrating tumor cell and stromal cell interactions. *Bone* 48:54–65
42. Nakamura I, le Duong T, Rodan SB, Rodan GA (2007) Involvement of alpha(v)beta3 integrins in osteoclast function. *J Bone Miner Metab* 25:337–344
43. Pecheur I, Peyruchaud O, Serre CM, Guglielmi J, Volland C, Bourre F, Margue C, Cohen-Solal M, Buffet A, Kieffer N, Clezardin P (2002) Integrin alpha(v)beta3 expression confers on tumor cells a greater propensity to metastasize to bone. *FASEB J* 16:1266–1268
44. Sloan EK, Pouliot N, Stanley KL, Chia J, Moseley JM, Hards DK, Anderson RL (2006) Tumor-specific expression of alphav-beta3 integrin promotes spontaneous metastasis of breast cancer to bone. *Breast Canc Res* 8:R20
45. Zhao Y, Bachelier R, Treilleux I, Pujuguet P, Peyruchaud O, Baron R, Clement-Lacroix P, Clezardin P (2007) Tumor alphavbeta3 integrin is a therapeutic target for breast cancer bone metastases. *Cancer Res* 67:5821–5830
46. Sterling JA, Guelcher SA (2011) Bone structural components regulating sites of tumor metastasis. *Curr Osteoporos Rep*
47. Van der Velde-Zimmermann D, Verdaasdonk MA, Rademakers LH, De Weger RA, Van den Tweel JG, Joling P (1997) Fibronectin distribution in human bone marrow stroma: matrix assembly and tumor cell adhesion via alpha5 beta1 integrin. *Exp Cell Res* 230:111–120
48. Takayama S, Ishii S, Ikeda T, Masamura S, Doi M, Kitajima M (2005) The relationship between bone metastasis from human breast cancer and integrin alpha(v)beta3 expression. *Anticancer Res* 25:79–83
49. Geiger B, Bershasky A (2002) Exploring the neighborhood: adhesion-coupled cell mechanotransducers. *Cell* 110:139–143
50. Moore SW, Roca-Cusachs P, Sheetz MP (2010) Stretchy proteins on stretchy substrates: the important elements of integrin-mediated rigidity sensing. *Dev Cell* 19:194–206
51. Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D, Hammer DA, Weaver VM (2005) Tensional homeostasis and the malignant phenotype. *Cancer Cell* 8:241–254
52. Paszek MJ, Weaver VM (2004) The tension mounts: mechanics meets morphogenesis and malignancy. *J Mammary Gland Biol Neoplasia* 9:325–342
53. Huang SJ, Ingber DE (2005) Cell tension, matrix mechanics, and cancer development. *Cancer Cell* 8:175–176
54. Reijnders CM, Bravenboer N, Tromp AM, Blankenstein MA, Lips P (2007) Effect of mechanical loading on insulin-like growth factor-i gene expression in rat tibia. *J Endocrinol* 192:131–140
55. Mantila Roosa SM, Liu Y, Turner CH (2011) Gene expression patterns in bone following mechanical loading. *J Bone Miner Res* 26:100–112
56. Hochmuth RM (2000) Micropipette aspiration of living cells. *J Biomech* 33:15–22
57. Jiang G, Huang AH, Cai Y, Tanase M, Sheetz MP (2006) Rigidity sensing at the leading edge through alphavbeta3 integrins and rtpalpha. *Biophys J* 90:1804–1809
58. Saez A, Buguin A, Silberzan P, Ladoux B (2005) Is the mechanical activity of epithelial cells controlled by deformations or forces? *Biophys J* 89:L52–L54
59. Discher DE, Janmey P, Wang YL (2005) Tissue cells feel and respond to the stiffness of their substrate. *Science* 310:1139–1143
60. Katsumi A, Orr AW, Tzima E, Schwartz MA (2004) Integrins in mechanotransduction. *J Biol Chem* 279:12001–12004
61. Harjanto D, Zaman MH (2010) Matrix mechanics and receptor-ligand interactions in cell adhesion. *Org Biomol Chem* 8:299–304
62. Luo BH, Carman CV, Springer TA (2007) Structural basis of integrin regulation and signaling. *Annu Rev Immunol* 25:619–647
63. Humphries JD, Byron A, Humphries MJ (2006) Integrin ligands at a glance. *J Cell Sci* 119:3901–3903
64. Berrier AL, Yamada KM (2007) Cell-matrix adhesion. *J Cell Physiol* 213:565–573
65. den Hertog J, Pals CE, Peppelenbosch MP, Tertoolen LG, de Laat SW, Kruijer W (1993) Receptor protein tyrosine phosphatase alpha activates pp 60c-src and is involved in neuronal differentiation. *EMBO J* 12:3789–3798
66. Na S, Collin O, Chowdhury F, Tay B, Ouyang M, Wang Y, Wang N (2008) Rapid signal transduction in living cells is a unique feature of mechanotransduction. *Proc Natl Acad Sci USA* 105:6626–6631
67. Zheng XM, Resnick RJ, Shalloway D (2000) A phosphotyrosine displacement mechanism for activation of src by ptpalpha. *EMBO J* 19:964–978
68. Schmidt C, Pommerenke H, Durr F, Nebe B, Rychly J (1998) Mechanical stressing of integrin receptors induces enhanced tyrosine phosphorylation of cytoskeletally anchored proteins. *J Biol Chem* 273:5081–5085
69. Kakonen ea SM (2005) Transforming growth factor beta stimulates parathyroid hormone related protein an osteolytic metastases via smad and mapk signaling pathways. *J Biol Chem* 227
70. Wang N, Butler JP, Ingber DE (1993) Mechanotransduction across the cell surface and through the cytoskeleton. *Science* 260:1124–1127
71. Galbraith CG, Yamada KM, Sheetz MP (2002) The relationship between force and focal complex development. *J Cell Biol* 159:695–705
72. Giannone G, Jiang G, Sutton DH, Critchley DR, Sheetz MP (2003) Talin1 is critical for force-dependent reinforcement of

- initial integrin-cytoskeleton bonds but not tyrosine kinase activation. *J Cell Biol* 163:409–419
73. von Wichert G, Jiang G, Kostic A, De Vos K, Sap J, Sheetz MP (2003) Rptp- α acts as a transducer of mechanical force on α 5 β 1-integrin-cytoskeleton linkages. *J Cell Biol* 161:143–153
 74. Choquet D, Felsenfeld DP, Sheetz MP (1997) Extracellular matrix rigidity causes strengthening of integrin-cytoskeleton linkages. *Cell* 88:39–48
 75. Zhao XH, Laschinger C, Arora P, Szasz K, Kapus A, McCulloch CA (2007) Force activates smooth muscle α -actin promoter activity through the rho signaling pathway. *J Cell Sci* 120:1801–1809
 76. Yeung T, Georges PC, Flanagan LA, Marg B, Ortiz M, Funaki M, Zahir N, Ming W, Weaver V, Janmey PA (2005) Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil Cytoskeleton* 60:24–34
 77. Zaman MH, Trapani LM, Sieminski A, MacKellar D, Gong H, Kamm RD, Wells A, Lauffenburger DA, Matsudaira P (2006) Migration of tumor cells in 3d matrices is governed by matrix stiffness along with cell-matrix adhesion and proteolysis. *PNAS* 103:10889–10894
 78. Alexander NR, Branch KM, Iwueke IC, Guelcher SA, Weaver AM (2008) Extracellular matrix rigidity promotes invadopodia activity. *Curr Biol* 18:1295–1299
 79. Engler AJ, Sen S, Sweeney HL, Discher DE (2006) Matrix elasticity directs stem cell lineage specification. *Cell* 126:677–689
 80. Enderling H, Alexander NR, Clark E, Branch KM, Estrada L, Croke C, Jourquin J, Lobdell N, Zaman MH, Guelcher SA, Anderson A, Weaver AM (2008) Dependence of invadopodia function on collagen fiber spacing and crosslinking: computational modeling and experimental evidence. *Biophys J* 95:2203–2218
 81. Smith KE, Hyzy SL, Sunwoo M, Gall KA, Schwartz Z, Boyan BD (2010) The dependence of mg63 osteoblast responses to (meth)acrylate-based networks on chemical structure and stiffness. *Biomaterials* 31:6131–6141
 82. Khatriwala CB, Kim PD, Peyton SR, Putnam AJ (2009) Ecm compliance regulates osteogenesis by influencing mapk signaling downstream of rhoa and rock. *J Bone Miner Res* 24:886–898
 83. Butcher DT, Alliston T, Weaver VM (2009) A tense situation: forcing tumour progression. *Nat Rev Cancer* 9:108–122
 84. Guelcher SA, Dumas J, Srinivasan A, Didier JE, Hollinger JO (2008) Synthesis, mechanical properties, biocompatibility, and biodegradation of polyurethane networks from lysine polyisocyanates. *Biomaterials* 29:1762–1775
 85. Gibson LJ, Ashby MF (1997) Cellular solids: structure and properties. Cambridge University Press, Cambridge
 86. Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C, Guise TA, Massague J (2003) A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* 3:537–549
 87. Huang JH, Peng XL, Xiong CY, Fang J (2011) Influence of substrate stiffness on cell–substrate interfacial adhesion and spreading: a mechano-chemical coupling model. *J Coll Interfac Sci* 355:503–508
 88. Bell GI (1978) Models for the specific adhesion of cells to cells. *Science* 200:618–627
 89. Bershadsky AD, Balaban NQ, Geiger B (2003) Adhesion-dependent cell mechanosensitivity. *Annu Rev Cell Dev Biol* 19:677–695
 90. Desgrosellier JS, Cheresh DA Integrins in cancer: biological implications and therapeutic opportunities. *Nat Rev Cancer* 10:9–22
 91. Engleman VW, Nickols GA, Ross FP, Horton MA, Griggs DW, Settle SL, Ruminski PG, Teitelbaum SL (1997) A peptidomimetic antagonist of the α 5 β 1 integrin inhibits bone resorption in vitro and prevents osteoporosis in vivo. *J Clin Invest* 99:2284–2292
 92. Galliher AJ, Schiemann WP (2006) β 3 integrin and src facilitate transforming growth factor- β mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. *Breast Cancer Res* 8:R42
 93. Matthews BD, Overby DR, Mannix R, Ingber DE (2006) Cellular adaptation to mechanical stress: role of integrins, rho, cytoskeletal tension and mechanosensitive ion channels. *J Cell Sci* 119:508–518
 94. Wang Y, Botvinick EL, Zhao Y, Berns MW, Usami S, Tsien RY, Chien S (2005) Visualizing the mechanical activation of src. *Nature* 434:1040–1045
 95. Sawada Y, Tamada M, Dubin-Thaler BJ, Cherniavskaya O, Sakai R, Tanaka S, Sheetz MP (2006) Force sensing by mechanical extension of the src family kinase substrate p130cas. *Cell* 127:1015–1026
 96. Huvneers S, Danen EH (2009) Adhesion signaling - crosstalk between integrins, src and rho. *J Cell Sci* 122:1059–1069
 97. Harms ea (2004) A small molecule antagonist of the α 5 β 1 integrin suppresses mda-mb-435 skeletal metastasis. *Clin Exp Met* 21:119–128
 98. Zhao YBR, Treilleux I, Pujuguet P, Peyruchaud O, Baron R (2007) Tumor α 5 β 1 integrin is a therapeutic target for breast cancer bone metastases. *Cancer Res* 67:5821–5830
 99. Myoui ANR, Williams PJ, Hiraga T, Tamura D, Michigami T et al (2003) C-src tyrosine kinase activity is associated with tumor colonization in bone and lung in an animal model of human breast cancer metastasis. *Cancer Res* 63:5028–5033
 100. Duband JL, Nuckolls GH, Ishihara A, Hasegawa T, Yamada KM, Thiery JP, Jacobson K (1988) Fibronectin receptor exhibits high lateral mobility in embryonic locomoting cells but is immobile in focal contacts and fibrillar streaks in stationary cells. *J Cell Biol* 107:1385–1396
 101. Felsenfeld DP, Schwartzberg PL, Venegas A, Tse R, Sheetz MP (1999) Selective regulation of integrin-cytoskeleton interactions by the tyrosine kinase src. *Nat Cell Biol* 1:200–206
 102. Zhang X, Jiang G, Cai Y, Monkley SJ, Critchley DR, Sheetz MP (2008) Talin depletion reveals independence of initial cell spreading from integrin activation and traction. *Nat Cell Biol* 10:1062–1068
 103. Galliher AJSW (2006) β 3 integrin and src facilitate transforming growth factor β mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. *Breast Cancer Res* 8:R42
 104. Gui GP, Wells CA, Yeomans P, Jordan SE, Vinson GP, Carpenter R (1996) Integrin expression in breast cancer cytology: a novel predictor of axillary metastasis. *Eur J Surg Oncol* 22:254–258
 105. Mizejewski GJ (1999) Role of integrins in cancer: survey of expression patterns. *Proc Soc Exp Biol Med* 222:124–138
 106. Shane E Evolving data about subtrochanteric fractures and bisphosphonates. *N Engl J Med* 362:1825–1827
 107. Coleman R, Woodward E, Brown J, Cameron D, Bell R, Dodwell D, Keane M, Gil M, Davies C, Burkinshaw R, Houston SJ, Grieve RJ, Barrett-Lee PJ, Thorpe H (2011) Safety of zoledronic acid and incidence of osteonecrosis of the jaw (onj) during adjuvant therapy in a randomised phase iii trial (azure: Big 01-04) for women with stage ii/iii breast cancer. *Breast Cancer Res Treat*
 108. Black DM, Kelly MP, Genant HK, Palermo L, Eastell R, Bucci-Rechtweg C, Cauley J, Leung PC, Boonen S, Santora A, de Papp A, Bauer DC (2010) Bisphosphonates and fractures of the

- subtrochanteric or diaphyseal femur. *N Engl J Med* 362:1761–1771
109. McClung MR (2006) Inhibition of rankl as a treatment for osteoporosis: preclinical and early clinical studies. *Curr Osteoporos Rep* 4:28–33
 110. Gonzalez-Suarez E, Jacob AP, Jones J, Miller R, Roudier-Meyer MP, Erwert R, Pinkas J, Branstetter D, Dougall WC (2010) Rank ligand mediates progesterin-induced mammary epithelial proliferation and carcinogenesis. *Nature* 468:103–107
 111. Biswas S, Guix M, Rinehart C, Dugger TC, Chytil A, Moses HL, Freeman ML, Arteaga CL (2007) Inhibition of tgf-beta with neutralizing antibodies prevents radiation-induced acceleration of metastatic cancer progression. *J Clin Invest* 117:1305–1313
 112. Ganapathy V, Ge R, Grazioli A, Xie W, Banach-Petrosky W, Kang Y, Lonning S, McPherson J, Yingling JM, Biswas S, Mundy GR, Reiss M (2010) Targeting the transforming growth factor-beta pathway inhibits human basal-like breast cancer metastasis. *Mol Cancer* 9:122
 113. Mohammad KS, Javelaud D, Fournier PG, Niewolna M, McKenna CR, Peng XH, Duong V, Dunn LK, Mauviel A, Guise TA (2010) The transforming growth factor- β receptor i kinase inhibitor sd-208 reduces the development and progression of melanoma bone metastases. *Cancer Res*
 114. Edwards JR, Nyman JS, Lwin ST, Moore MM, Esparza J, O'Quinn EC, Hart AJ, Biswas S, Patil CA, Lonning S, Mahadevan-Jansen A, Mundy GR (2010) Inhibition of tgf- β signaling by 1d11 antibody treatment increases bone mass and quality in vivo. *J Bone Miner Res* 25:2419–2426
 115. Mohammad KS, Chen CG, Balooch G, Stebbins E, McKenna CR, Davis H, Niewolna M, Peng XH, Nguyen DH, Ionova-Martin SS, Bracey JW, Hogue WR, Wong DH, Ritchie RO, Suva LJ, Derynck R, Guise TA, Alliston T (2009) Pharmacologic inhibition of the tgf-beta type i receptor kinase has anabolic and anti-catabolic effects on bone. *PLoS One* 4:e5275
 116. Tan AR, Alexe G, Reiss M (2009) Transforming growth factor-beta signaling: emerging stem cell target in metastatic breast cancer? *Breast Canc Res Treat* 115:453–495
 117. Barman SA, Zhu S, White RE (2009) RhoA/rho-kinase signaling: a therapeutic target in pulmonary hypertension. *Vasc Health Risk Manag* 5:663–671
 118. Olson MF (2008) Applications for rock kinase inhibition. *Curr Opin Cell Biol* 20:242–248
 119. Summy JM, Gallick GE (2006) Treatment for advanced tumors: Src reclaims center stage. *Clin Canc Res* 12:1398–1401
 120. Boyce BF, Xing L, Yao Z, Yamashita T, Shakespeare WC, Wang Y, Metcalf CA 3rd, Sundaramoorthi R, Dalgarno DC, Iulucci JD, Sawyer TK (2006) Src inhibitors in metastatic bone disease. *Clin Canc Res* 12:6291s–6295s
 121. Desgrosellier JSCD (2010) Integrins in cancer: biological implications and therapeutic opportunities. *Nat Rev Cancer* 10:9–22
 122. Ruppender NS, Merkel AR, Guelcher SA (Submitted) Bone matrix rigidity stimulates signaling through $\alpha v \beta 3$, src and mapk to induce the expression of pthrp in osteolytic tumor cells
 123. Brooks PC, Stromblad S, Klemke R, Visscher D, Sarkar FH, Cheresch DA (1995) Antiintegrin $\alpha v \beta 3$ blocks human breast cancer growth and angiogenesis in human skin. *J Clin Invest* 96:1815–1822
 124. Moschos SJ, Sander CA, Wang W, Reppert SL, Drogowski LM, Jukic DM, Rao UN, Athanassiou C, Buzoianu M, Mandic M, Richman L, McKinney L, Leininger J, Tice DA, Hammershaimb L, Kirkwood JM (2010) Pharmacodynamic (phase 0) study using etaracizumab in advanced melanoma. *J Immunother* 33:316–325
 125. O'Day SJ, Pavlick AC, Albertini MR, Hamid O, Schalch H, Lang Z, Ling J, Mata M, Reddy M, Foster B (2011) Clinical and pharmacologic evaluation of two dose levels of intetumumab (cnto 95) in patients with melanoma or angiosarcoma. *Invest New Drugs*
 126. Stupp R, Hegi ME, Neyns B, Goldbrunner R, Schlegel U, Clement PM, Grabenbauer GG, Ochsenbein AF, Simon M, Dietrich PY, Pietsch T, Hicking C, Tonn JC, Diserens AC, Pica A, Hermisson M, Krueger S, Picard M, Weller M (2010) Phase i/ii study of cilengitide and temozolomide with concomitant radiotherapy followed by cilengitide and temozolomide maintenance therapy in patients with newly diagnosed glioblastoma. *J Clin Oncol* 28:2712–2718
 127. Lauth M, Bergstrom A, Shimokawa T, Toftgard R (2007) Inhibition of gli-mediated transcription and tumor cell growth by small-molecule antagonists. *Proc Natl Acad Sci USA* 104:8455–8460
 128. Galliher AJ, Schiemann WP (2007) Src phosphorylates tyr284 in tgf-beta type ii receptor and regulates tgf-beta stimulation of p38 mapk during breast cancer cell proliferation and invasion. *Cancer Res* 67:3752–3758