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Genetics of breast cancer bone metastasis: a sequential multistep pattern

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Abstract Bone metastasis accounts for the vast majority of breast cancer (BC) metastases, and is related to a high rate of morbidity and mortality. A number of seminal studies have uncovered gene expression signatures involved in BC development and bone metastasis; each of them points at a distinct step of the 'invasion-metastasis cascade'. In this review, we provide most recently discovered functions of sets of genes that are selected from widely accepted gene signatures that are implicate in BC progression and bone metastasis. We propose a possible sequential pattern of gene expression that may lead a benign primary breast tumor to get aggressiveness and progress toward bone metastasis. A panel of genes which primarily deal with features like DNA replication, survival, proliferation, then, angiogenesis, migration, and invasion has been identified. TGF-β, FGF, NFκB, WNT, PI3K, and JAK-STAT signaling pathways, as the key pathways involved in breast cancer development and metastasis, are evidently regulated by several genes in all three signatures. Epithelial to mesenchymal transition that is also an important mechanism in cancer stem cell generation and metastasis is evidently regulated by these genes. This review provides a comprehensive insight regarding breast cancer bone metastasis that may lead to a better understanding of the disease and take step toward better treatments.

Keywords Breast cancer metastasis · Bone metastasis · Gene signature · Signaling network

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Metastasis, a complex multistep event

Breast cancer (BC) metastasis is the leading cause of cancer related deaths among women (metastasis; the spread of tumor cells from primary site to distant organs) [1, 2]. More than 70 % of BC patients in advanced stage develop bone metastases which are related to high rate of morbidity and mortality [3, 4]. The vast majority of researchers have focused to identify the underlying molecular and cellular mechanisms of this complex phenomenon. Developing gene expression signatures, involved in certain stages of BC metastasis, are among seminal discoveries that may let to understand the basics involved in the 'invasion-metastasis cascade', and consequently the generation of effective therapies against metastasis.

'Invasion-metastasis cascade' represents a multistep process that consists a set of sequential events, in which tumor cells invade from primary site, disseminate through circulation, and reconstitute secondary tumors at distant tissue(s) [5]. It has been investigated that breast tumor cells encounter a plenty of changes when disseminating from primary to distant sites, including changes in gene expression pattern [6], and in the states of stemness [7], that define clonal evolution and cancer stem cell (CSC) theories, respectively. Genetic alterations and subsequent shift in cellular events and states must be considered in the context of different microenvironments in different steps of metastasis in order to uncover mysteries of the complex process of metastasis cascade.

Clonal evolution and CSC theories are two models for tumor heterogeneity, cancer development, and metastasis that are widely accepted [8, 9]. Clonal evolution theory indicates that different lineages of cancer cells are developed during multistep genetic and epigenetic alterations, which lead to acquiring different features required for

tumor development and metastasis [10]. On the other hand, CSCs, which have features of self-renewal, tumorigenesis, multilineage differentiation, motility, invasiveness and apoptosis resistance, are believed to be required for the development and maintenance of several forms of human cancers, including BC. Based on the CSC theory, tumor cells are not naturally alike, based on the state of stemness, and are organized in a hierarchical pattern in which CSCs are considered to be at the top of the apex [11–13]. It is believed that both models of clonal evolution and CSC can be applied in cancer development and metastasis, and that tumors heterogeneity can be generated from both of them [8].

The pattern of gene expression is likely the most critical determinant of CSC state. A quite sophisticated program that leads to expression of a group of genes, and simultaneously suppression of others, directs all features of a tumor cell in a precise time. Gene expression signatures have been developed since more than a decade for a variety of diseases like cancer metastasis. Gene signatures that have been developed for BC metastasis are ostensibly behind-the-scene forces of cellular events. In this study, we provide most recently discovered functions of sets of genes that are selected from seminal widely accepted gene signatures. We then propose a possible sequential pattern of gene expression that may lead to a benign primary breast tumor to get aggressiveness and progress toward bone metastasis. We also discuss most prominent molecular mechanisms involved in BC bone metastasis.

Gene signatures associated to breast cancer progression and bone metastasis

A number of seminal studies have uncovered gene expression signatures involved in BC development and metastasis; each of them points at a distinct step of the 'invasion-metastasis cascade' (Tables 1, 2, 3, 4) [14–16]. Today, these findings have entered to the diagnosis as predictors of disease outcome in BC patients [17]. Particularly, such discoveries have heralded the new era of personalized medicine, while predicting the clinical outcome of patients based on a set of distinct gene expression patterns [18]. Although improving, our understanding of the exact molecular and, most importantly, cellular mechanisms of BC metastasis is poor, and therefore reliable treatments are lacking. Analysis of data resulting from high throughput genome wide assays, and translation of the molecular pattern to cellular mechanisms/pathways may provide novel perspective to understand the complex nature of metastasis, and subsequently develop new therapeutic strategies. Among studies that have provided gene signatures for BC progression and bone metastasis, the ones by van't Veer et al., Smid et al., and Kang et al. are of most seminal and widely accepted.

Table 1 Gene signatures associated to breast cancer metastasis

Gene signature (authors)	Type of study	Year of study
Perou, C.M. et al.	In vitro, and in vivo (human primary breast tumor)	1999
Perou, C.M. et al.	In vivo (human primary breast tumor)	2000
Zajchowski, D.A. et al.	In vitro	2001
Sorlie, T. et al.	In vivo (human primary breast tumor)	2001
West, M. et al.	In vivo (human primary breast tumor)	2001
van't Veer, L. J. et al.	In vivo (human primary breast tumor)	2002
Kang, Y. et al.	In vitro, and in vivo (mouse metastatic bone metastatic tumor)	2003
Smid, M. et al.	In vivo (human primary breast tumor)	2006

van't Veer's signature

Several studies regarding gene expression pattern in BC have been developed since more than a decade (Table 1). Earliest ones [19–23] were not quite sufficient to be utilized for predictive and therapeutic purposes. This may be because of the inconsistency in different studies (e.g., different kinds of primary tumors), and abundance of heterogeneity in tumors. The first highly applicable reported gene signature, by van't Veer and colleagues (*van't Veer's signature*), have established a 70-gene prognosis profile from primary tumors of young BC patients. (Table 2 comprises a panel of selected genes from *van't Veer's signature*, based on their significant expression in most poor prognosed patients, and the status of being well studied). They identified a set of genes strongly predicting distant metastasis in patients who were lymph node negative, called poor prognosis signature [14]. Their finding uncovered a pattern of gene expression required for primary tumor cells to become invasive; capable to evade from primary site.

van't Veer's signature comprises genes associated to a well-orchestrated program for the regulation of different features required for primary tumors to grow and escape from primary site, including cell cycle, DNA replication, proliferation, tumorigenesis, survival, angiogenesis, migration, and invasion (Table 2). In particular, among those, *CCNB2*, *CCNE2*, *MCM6*, *TSPYL5*, *NUSAP1*, *CMC2*, *ECT2*, *ORC6*, *DTL*, *PRC1*, *MELK*, *EGLN1*, *SLC2A3*, *RAB6B*, *ESM1*, *RAD21*, *CDC25B*, *CDK16*, *CENPA*, *PGK1*, *MAD2L1*, *CKS2*, *BUB1*, *FGF18*, *WISP1*, and *IGFBP5* are known regulators of cell cycle, DNA

Table 2 van't Veer's signature of breast cancer poor/good prognosis genes

Gene symbol	Gene description	Functions and features
Cell cycle/DNA replication/proliferation/tumorigenesis/survival		
CCNB2	Cyclin B2	Cell cycle control [24], Serves as oncogene, and potential biomarker of unfavorable prognosis [125], TGF-β-mediated cell cycle control [27]
CCNE2	Cyclin E2	Cell cycle control [24], resistance to both endocrine therapy and CDK4 inhibition [126], increase in pathogenesis and kinase activity [127]
MCM6	Mini-chromosome maintenance proteins	DNA replication, and growth [25]
TSPYL5	TSPY-like 5	Suppression of p53 [128], cell growth via regulation of p21 and PTEN/AKT pathways [129]
NUSAP1	Nucleolar and spindle associated protein 1	Activation by E2F1 [130], spindle organization in mitosis thorough cdk1 activation [131, 132]
CMC2	COX assembly mitochondrial protein 2 homolog	Cytochrome C oxidase biogenesis [133]
ECT2	Epithelial cell transforming sequence 2 oncogene	Cell cycle progression and proliferation [134], mesenchymal amoeboid transition [135] and migration [136]
ORC6	Origin recognition complex, subunit 6	Proliferation [137]
DTL	Denticleless E3 ubiquitin protein ligase homolog	Tumor growth [138], proliferation, survival, and metastasis [139, 140]
PRC1	Protein regulator of cytokinesis 1	Proliferation [141]
MELK	Maternal embryonic leucine zipper kinase	Proliferation of tumor and CSCs, and inhibition of apoptosis [142–145]
EGLN1	egl-9 family hypoxia-inducible factor 1	Proliferation, survival [146], target of HIF [147], involved in the processing of TGF-β1 [28], angiogenesis [148], maintenance of epithelial differentiation [149]
SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	Preferential glucose uptake in TICs [150], induced by HIF [151]
IGFBP5	Insulin-like growth factor binding protein 5	Cell survival, cell adhesion through activation of integrin-linked kinase (ILK) and Akt, and reduces migration [152]
RAB6B	Member of RAS oncogene family	Tumor proliferation, malignant transformation [153]
ESM1	Endothelial cell specific molecule-1	Tumor growth, and metastasis through NFκB pathway [31], angiogenesis [154]
RAD21	Double-strand-break repair protein rad21 homolog	Sister chromatid cohesion in mitotic cells [155], DNA repair [156], and estrogen-mediated regulation of MYC [36]
CDK16	Cyclin dependent protein kinases	Cell proliferation [157]
CDC25B	Cell division cycle 25B	Cell proliferation [158], overexpression of c-myc [37]
CENPA	Centromere protein A	Tumor progression, cell cycle regulation, survival [159], chromosomal instability [160]
PGK1	Phosphoglycerate kinase 1	Increase in the expression of CXCR4, angiogenic switch, tumor growt h [45], target of PPARγ (cell proliferation) [161], and induce bone metastasis [44]
MAD2L1	Mitotic arrest-deficient 2 L1	Cell proliferation [162], tumorigenesis [163]
CKS2	Cdc28 kinase subunit 2	Tumorigenesis, proliferation, apoptosis resistance [164, 165]
BUB1	Mitotic checkpoint serine/threonine kinase	Cell proliferation [166, 167]
Angiogenesis/migration/invasion		
FLT1	Vascular endothelial growth factor receptor	Angiogenesis and vasculogenesis [168, 169], formation of premetastatic niche and bone directed metastasis of breast cancer [43]
EXT1	Exostosin glycosyltransferase 1	Bone development [170], migration [171]
DIAPH3	Diaphanous-related formin 3	Motility/migration and the formation of filopodium in tumor [172–174]
CDC42BPA	Serine/Threonine protein kinase	Tumor cell invasion [175], migration [176]
EXOC7	Exocyst complex component 7	Angiogenesis [177], migration [178]
NMU	Neuromedin U	Tumorigenicity, metastasis [179], cell migration, invasiveness, tumor cell dissemination [180]
VEGF	Vascular endothelial growth factor	Angiogenesis [181, 182], metastasis [183], EMT [41]
MMP9	Matrix metalloproteinase	Induction of VEGF [184, 185], EMT, invasion and metastasis [39, 40]
FGF18	Fibroblast growth factor 18	Cell survival [186], growth, migration, invasion through NFκB pathway, angiogenesis [32, 187]
WISP1	WNT1 inducible signaling pathway protein 1	WNT1/β-catenin responsive oncogene [188], survival [189], induction of the expression of VCAM1 [190], increase the expression of MMP2 and migration through FAK, MEK, ERK, p65 and NF-κB pathways [33], involved the regulation of TGF-β1 to control osteoblast function [191], metastasis to bone [192]
TGFB3	Transforming growth factor, beta 3	Induction of breast CSCs [30], involved in the induction of MMPs, EMT, and invasion through MAPK pathway [29], induced by HIF-1 [193], induced by Snail/Slug/β-catenin-TCF4 pathway [194], induction of apoptosis [195]

Table 3 Smid's signature of breast cancer bone metastasis genes

Gene symbol	Gene description	Functions and features
TFF1	Trefoil factor-1	Oncogenicity [196], cell survival, anchorage-independent growth [52], angiogenesis, migration, and invasion [51], protect the mucosa from insults, stabilize the mucus layer and affect healing of the epithelium [197]
TFF3	Trefoil factor-3	Tumor progression and dissemination [198], regulation of VEGF expression induced by hypoxia [55], protect the mucosa from insults, stabilize the mucus layer and affect healing of the epithelium [197]
AGR2	A protein disulfide isomerase; anterior gradient homolog 2	Tumor growth, cell migration, cellular transformation [199], cancer cell survival [200], metastasis [201], and production of intestinal mucus [202]
NAT1	Arylamine N-acetyltransferase-1	Cell growth, anchorage independent growth, E-cadherin (cell–cell contact) inhibition, and invasion [203, 204]
CRIP1	Cysteine-rich intestinal protein-1	Cellular growth and differentiation [205]
RND1	Member of the Rho GTPase family	Disassembly of actin filament structures, and loss of cell adhesion [206]
TSPAN1	Tetraspanin 1	Cell growth, migration, and invasion [207, 208]
FGFR3	Fibroblast growth factor receptor 3	Cellular proliferation, survival, migration, angiogenesis [97]
SCUBE2	Signal peptide CUB (complement proteins C1r/C1 s, Uegf, and Bmp 1)-EGF domain-containing protein2	Growth inhibitory effects, antagonizing bone morphogenetic protein, and suppressing the β-catenin pathway (interacting with E-cadherin) [63, 209], enhance the Sonic Hedgehog signaling activity [210]
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6	Tumorigenesis, disruption of cell polarity, and anoikis resistance [211, 212]
TOM1L1	Target of Myb-1 Like	Enhancement of IL2-Jak2-STAT3 signaling pathway [213], and involvement of EGF signaling [214]
KRT16	Keratin 16	Migration and invasion [215]
FGFBP1	Fibroblast growth factor binding protein1	Angiogenesis, enhancement in FGF-1- and FGF-2-dependent proliferation, FGF-2-induced ERK activation, and migration [59, 216, 217]
FOXO3A	Forkhead box class O3A transcription factor	Suppression of tumor cells; Regulation the expression of p27/Kip1, cyclin D1, and cyclin E, induction of apoptosis through Bim and FasL [218–222], regulation of glycolysis downstream of Akt through transcriptional control of Tsc1 [223], and repression of ER-α [224], and estrogen-dependent proliferation [225], repression of VEGF [226], promotion of invasive migration through MMP9 and MMP-13 upregulation [62]
KRT6B	Keratin 6B	Migration and invasion [215]
SNAI1	Snail transcription factor	Induction of EMT, invasion, and metastasis [57] by suppressing E-cadherin [227], and cell cycle, and induction of apoptosis resistance [228]
TMSB15A	Thymosin beta 15a	Tumor progression and metastasis [229], motility [230]

replication, proliferation, and survival [24–26]. Upregulation of these genes can be considered as the first requirements of primary tumor for its growth in order to be prepared for dissemination. Afterward, *ECT2*, *EGLN1*, *ESM1*, *FLT1*, *EXT1*, *DIAPH3*, *EXOC7*, *NMU*, *CDC42BPA*, *VEGF*, *MMP9*, *FGF18*, *WISP1*, and *TGF-β3*, are well-known pivotal elements that participate in angiogenesis, migration, and invasion. Tumor cells which express these genes seem to be adept for invasion from primary site (please see Table 2 for detail functions of gene products).

A variety of key molecular mechanisms are regulated by *van't Veer's signature* gene products. Remarkably, TGF-β signaling pathway likely plays important roles in the regulation of cell cycle, proliferation, induction of EMT, CSCs, and MMPs [27–30] (Table 2). Furthermore, NFκB signaling is also involved in this level of tumor progression taking part in tumor growth and metastasis by the mediation of *ESM1, FGF18,* and *WISP1* [31–33]. In this step, the transcription factor MYC, which is well known for its association to breast tumor proliferation [34, 35], may also play important roles as is shown to be regulated by at least two of *van't Veer's signature* genes including *RAD21* and *CDC25B* [36, 37]. Notably, '*epithelial to mesenchymal transition*' (EMT), a process that is shown to be essential

Table 4 Kang's signature of breast cancer bone metastasis genes

Gene symbol	Gene description	Functions and features
Angiogenesis, migration/invasion, EMT		
MCAM	Melanoma cell adhesion molecule	Migration, invasion, tumorigenicity [231], motility [232], angiogenesis [233, 234], EMT [232, 235], and metastasis [236]
PTK7	Protein tyrosine kinase 7	Cell cycle regulation, EMT [237], motility [238], regulation of WNT/planar cell polarity pathway [239], invasion, angiogenesis [240], and target of MMP14 [241]
RGCC	Regulator of cell cycle	Induction of EMT, migration, and invasion via NFκB signaling [242], interact with Smad3 to promote TGF-β1 mediated EMT [65, 69]
CTGF	Connective Tissue Growth factor	Induction of EMT-like cell fate [243], protection of the vasculature [244], hypoxia induced [245] angiogenesis [246], migration and invasion [247] through MMP-13 upregulation, and FAK, ERK, NFκB pathways [79], mediation of Smad-dependent TGFβ signaling to regulate mesenchymal cell proliferation [71], involve in bone metastasis [80, 84], and upregulated by EGF [248]
FGF5	Fibroblast growth factor-5	Autocrine and paracrine dependent cell growth, enhance the MAPK signaling [78], angiogenic and mitogenic factor [249, 250], survival and migration [251]
ADAMTS1	A disintegrin and metalloprotease with thrombospondin motifs protein 1	Tumor growth and metastasis [252], migration [253, 254], endothelial invasion [255], inhibition of angiogenesis [256, 257], induce bone metastasis [81]
CXCR4	Chemokine (C-X-C motif) receptor 4	Modulating the trafficking of both cancer and normal stem cells [258], induction of bone metastasis [82], survival, proliferation, angiogenesis [259], migration, invasion through WNT/β-catenin pathway [260], upregulation/activation by TGF-β1 [72, 73], HIF [261], and Akt [262], and BMP4 [263], induces EMT [264], induces and maintains stemness in cancer stem cells [265]
IL11	Interleukin-11	TGF-β dependent [15, 74, 75] bone metastasis [74, 83–85], motility [266], and invasion via PI3K, Ras, STAT3, MAPK, and JNK mediation [267–269], induce production of RANKL [3]
MMP1	Matrix metalloproteinase-1	Tumorigenesis [270], induce bone metastasis [81], invasion, release IGF, FGF and TGF-β [76], vascular remodeling [271], induced by WNT signaling [272], MAPK [273], and BMP4 [263], activates PAR1 [270], roles in tumor extravasation [274]
Growth/angiogenesis inhibition		
FHL1	Four and a half LIM protein 1	Interaction with oestrogen receptors (ERs), breast cancer cell growth regulation [275], interaction with Smads 2,3,4 and suppress tumor growth and migration [276], suppression of VEGF [277]
DUSP1	Dual specificity phosphatase 1	Target of TGF-β1, inhibition of p38/MAPK and JNK [68, 278, 279]. Target of p53 and triggers apoptosis [280, 281], and involved in the auto-regulation of VEGF [282]
SOCS2	Suppressor of cytokine signaling 2	Suppression of proliferation and growth [283], prolactin-induced mammary gland development [284], enhance IL-2 and IL-3 signaling [285]
FST	Follistatin	Stimulation by TGF-β2 [70] and BRCA1 [286], Inhibition of BMPs [287–289], inhibition of activin [290], increase mTOR signaling via Smad3 [77], downstream of WNT4 signaling [291], inhibition of angiogenesis [292]
Survival and microenvironmental factors		
SLC4A7	Solute carrier family 4, sodium bicarbonate cotransporter, member 7	Major determinant of pH(i) in breast cancer primary tumor and metastasis [293]
NCF2	Neutrophil cytosolic factor 2	Target of p53 in activation of ROS and inhibition of apoptosis [294]

for tumor dissemination and metastasis of breast carcinomas [38], is enhanced through at least two of genes in this signature including *MMP9* and *VEGF*. These two factors play determining roles in preparing a hospitable microenvironment in which emitted signals trigger EMT in order to induce/maintain CSCs [39–42]. On the other hand, two of genes in this panel may tend to trigger primary tumor cells to metastasize to bone, including FLT1 and PGK1 [43, 44]. PGK1 increases the expression of CXCR4, which is one of the most important bone metastasis factors [45]. FLT1 also provide a premetastatic niche in bone and direct bone metastasis of BC [43]. Together, above information suggest the involvement of key regulators like TGF-β, NFκB, and MYC, as well as the process of EMT in first steps of BC metastasis within the primary tumor.

Biological functions of genes in *van't Veer's signature* define the hallmarks of cancer. Tian and colleagues have recently shown that *van't Veer's signature* gene products

functionally meet all the six *hallmarks of cancer* defined by Hanahan and Weinberg, including sustained proliferation, anti-growth signaling evasion, cell death resistance, immortality, angiogenesis, and invasion/metastasis [46]. They identified interconnected networks and showed that these genes are regulated by key tumorigenic factors like TP53, RB1, MYC, JUN and CDKN2A [47]. Interestingly, *van't Veer's signature* may also reflect the two additional hallmarks of next generation, including reprogramming of energy metabolism and evading immune destruction [1].

Adjustments of energy metabolism in order to fuel cell growth and division, is of most important features that lead to uncontrolled proliferation in neoplasms. Glycolytic fueling has been shown to be one of the most essential mechanisms in the reprogramming of energy, and associated with activated oncogenes like RAS, MYC, mutant tumor suppressors like TP53, certain signaling pathways like PI3K/Akt/PTEN, and hypoxia inducible factor 1(HIF1) [48, 49]. Now, as Tian and colleagues showed, and also from functions of certain genes including *TSPYL5*, *CMC2*, *CDC25B*, *EGLN1*, *SLC2A3*, *RAB6B*, and *TGF-β3* (see details of functions in Table 2), *van't Veer's signature* likely associates with the seventh hallmark of cancer, reprogramming of energy metabolism. As for the eighth hallmark, evading immune destruction, it has been shown that tumors that produce transforming growth factor (TGF)-β escape from immune surveillance, mainly by selective and direct suppression of the T cell cytotoxic gene responses [50]. Intriguingly, TGF-β signaling is undeniably of key factors in *van't Veer's signature*. Together, it seems that functions of gene products of *van't Veer's signature* also meet the two additional next generation hallmarks of cancer, in addition to the first six ones.

Smid's signature

Focusing on bone metastasis, Smid and colleagues have established a panel of genes in BC patients that are implicated to bone relapse (*Smid's signature*). They analyzed primary tumors of lymph node negative BC patients who, subsequently, had developed metastases. A set of 69 genes was identified to be differentially expressed in patients who had experienced bone metastasis versus patients with metastasis to other sites. (Table 3 comprises a panel of selected genes from *Smid's signature* that are significantly overexpressed and have been better studied). Notably, they developed classifier of tumors that metastasize to bone that was applicable in clinic [16].

Smid's signature provides a pattern of gene expression in primary tumors that obligates them to metastasize to bone. Genes in *Smid's signature* participate in essential features of metastasis including tumor growth, proliferation, survival, angiogenesis, migration, and invasion (Table 3). Among those, *TFF1*, *TFF3*, *AGR2*, *NAT1*, *CRIP1*, *TSPAN1*, *FGFR3*, *CEACAM6*, and *TMSB15A* may be categorized to play roles in cell growth/proliferation, and survival. Importantly, a number of genes in this signature take part in angiogenesis that include *TFF1*, *TFF3*, *FGFR3*, and *FGFBP1*. Thereupon, the vast majority of the genes in this panel which have seminal roles in migration and invasion are reported to be *TFF1*, *TFF3*, *AGR2*, *NAT1*, *RND1*, *TSPAN1*, *FGFR3*, *CEACAM6*, *KRT16*, *FGFBP1*, *FOXO3A*, *KRT6B*, and *SNAI1*. Interestingly, *TFF1*, *TFF3*, and *FGFR3* are present in all above categories, and seemingly play pivotal roles in the process of BC metastasis to bone.

Well-characterized crucial molecular mechanisms are controlled by *Smid's signature* gene products. Trefoil factor-1 (TFF1), that was the most differentially expressed gene associated to bone metastasis in *Smid's signature*, is ascertained to play roles in cell survival, anchorage-independent growth, angiogenesis, migration, and invasion in breast (and also other) tumor cells [51–54]. TFF3 induces angiogenesis by the regulation of VEGF, under the control of hypoxia [55]. AGR2, NAT1, FGFR3, and TSPAN1 have shown to play roles in tumor growth/proliferation/survival and/or migration/invasion. CRIP1, CEACAM6, and TMSB15A regulate tumor growth/proliferation and survival. RND1, KRT16, KRT6B, FOXO3A, and SNAI1 induce migration/invasion. It should be noted that, SNAI1 (also SNAIL) has determined to play pivotal roles as a master regulator of EMT [38, 56–58]. On the contrary, *SCUBE2*, and *FOXO3A* have generally shown to encode suppressors of tumor growth/proliferation, inducers of apoptosis, and repressor of angiogenesis. SCUBE2, and FOXO3A, although, are distinguished to play roles in line with induction of invasiveness, and metastasis, by regulation of Hedgehog signaling and matrix metalloproteinases, respectively [59–62] (Table 3).

Critical signaling pathways are associated with the genes in the *Smid's signature*. Apparently, *fibroblast growth factor* (FGF) signaling takes fundamental parts in this circuit, as *FGFR3* and *FGFBP1* that are directly linked to this pathway are two important overexpressed genes in this panel. ERK signaling is likely involved through activation by FGFBP1 [59]. In addition, *epidermal growth factor* (EGF) and *Janus kinase-signal transducers and activators of transcription* (JAK-STAT) signaling pathways are demonstrated to be enhanced by TOM1L1 [63]. Together, from the above mentioned findings, it seems that the genes in *Smid's signature* direct a complex program in which tumor cells proliferate and survive, then further induce and maintain angiogenesis, and finally enhance migration and invasion. Passing these two steps of gene expression (*van't Veer's and Smid's signatures*), tumor cells are capable to invade, intravasate, and survive in

circulation, heading to bone. These two sets of genes may be regulated in an overlapped, or in a sequential pattern.

Kang's signature

Focused on the gene expression pattern of breast tumor cells heading to bone, Kang and colleagues investigated a multigenic program in highly aggressive osteolytic BC metastatic cells (*Kang's signature*) (Table 4 comprises a panel of selected genes from *Kang's signature* that are significantly overexpressed and have been better studied) [15]. Their work provided a framework for the identification of genes mediating metastasis to different organs. Although *Kang's signature* was established in animal model, it has recently been confirmed in human BC patients as well [64]. These genes mostly encode secreted and cell membrane proteins, and are associated to the preparation of a compatible metastatic niche.

Kang's signature provides a panel of genes that may be categorized into four groups including: angiogenesis, migration/invasion, EMT, and growth/angiogenesis inhibition. Of those, *MCAM*, *PTK7*, *CTGF*, *FGF5*, and *CXCR4* play critical roles in angiogenesis. *MCAM*, *PTK7*, *RGCC*, *CTGF*, *FGF5*, *ADAMTS1*, *CXCR4*, *IL-11*, and *MMP1* are well-known essential factors for tumor migration/invasion. Notably, a number of key genes in this signature encode important inducers of EMT and CSC features including: MCAM, PTK7, RGCC, CTGF, and CXCR4. RGCC and Smad3 direct the induction of EMT through regulation of SNAIL and SLUG EMT transcription factors [65]. CXCR4 activates several signaling pathways, including AKT [66], a process in which Src plays a critical role [67]. It should be noted that, some genes in *Kang's signature* controversially function against tumor growth or angiogenesis (fourth group). Those include *FHL1*, *DUSP1*, *SOCS2*, *FST*, and *ADAMTS1* (see below). *SLC4A7* and *NCF2*, which are not categorized in these groups, play roles in preparing the microenvironment and inhibition of apoptosis, respectively (Table 4). From the above mentioned information, MCAM, PTK7, CTGF, and CXCR4 are categorized into all first three groups, and likely play critical roles in the last steps of BC bone metastasis.

Genes in *Kang's signature* function toward controlling principal signaling pathways, and govern a sophisticated signaling network that leads to a successful metastasis. Table 4 demonstrates undeniable deviation in the regulation of key signaling pathways, like TGF-β, WNT, NFκB, FGF, and MAPK, as underpinning functions of genes associated to bone metastasis. Almost half of the genes in this panel, including DUSP1, RGCC, FST, CTGF, CXCR4, IL-11, and MMP-1, are directly linked to the TGF-β signaling [65, 68–76]. FST also increase mTOR signaling via Smad3 [77]. PTK7, FST, CXCR4, and MMP-1 take part in the regulation of WNT signaling, and remarkably, RGCC and CTGF function via NFκB pathway. FGF5 mediate FGF signaling, and enhance MAPK pathway [78]. CTGF also act through ERK and FAK pathways [79]. Importantly, distinct gene expression pattern of *Kang's signature* specifically direct disseminated tumor cells to overt bone metastasis. CTGF, ADAMTS1, CXCR4, IL-11, and MMP1 are considered crucial inducers of bone metastasis [80–85]. Together, it seems that for a successful bone metastasis such sophisticated signaling network is required, which is controlled by the power of gene expression regulation.

Breast cancer bone metastasis; a multistep cascade of events

Several studies have reported genes that are associated with BC bone metastasis, from which some prognostic tools are provided in order to obtain best available treatments for individual BC patients. However, a comprehensive understanding of the nature of metastasis is yet to be investigated. Accordingly, due to the short knowledge of this complex phenomenon, well-suited therapies are lacking. In this review, we aimed to use published gene signatures, which have been shown to be significantly linked to progression and metastasis of BC to bone, to unmask a sequential pattern of gene expression that leads to colonization of breast tumors in bone.

Primary breast tumors cells ought to arrange a programmed gene expression pattern for their growth, survival, and invasion. Therefore, programs related to cell cycle progression, proliferation, apoptosis resistant, angiogenesis, invasion, and distant colonization need to be directed by nucleus. Genes in *van't Veer's* and *Smid's* signature seem to provide such well-coordinated program for tumor cells, as both signatures are derived from primary tumors of BC patients, who developed metastasis (in general) and bone metastasis, respectively [14, 16]. We thought that genes in these two signatures likely express in a time period that primary tumor: first get committed to metastasize, and second committed to metastasize to bone. Then, as is obvious in Tables 2 and 3, primary tumor first get ready for metastasis, and then get features of invasion, and intravasation. Tumor cells, then, acquire bone specific directing features of homing, extravasation, micro-colonization, and eventually macro-colonization (metastatic colonization) from the genes in *Kang's signature* (Table 4) (Fig. 1). This is likely because *Kang's signature* is primarily derived from metastatic breast tumor cells from bone lesions [15]. Importantly, key genes in *Kang's signature* including *MMP-1*, *CXCR4*, *FGF5* and *CTGF*, which was initially determined from study on metastatic MDA-MB-231 cells and mouse model, have been recently

Fig. 1 Sequential gene expression pattern, from primary tumor to metastatic colonization. *van't Veer's*, *Smid's*, and *Kang's* gene signatures shows a sequential pattern of expression that leads to breast cancer progression, metastasis. Genes in *van't Veer's* signature are mostly involved in the regulation of cell cycle, DNA replication, Proliferation, tumorigenesis, and survival, therefor, are essential for primary tumor cells to be prepared for invasion and metastasis. On the other hand some genes in this signature act toward angiogenesis, migration, and invasion. *ESM1*, *ECT2*, and *EGLN1* are present in both categories and likely play important roles in primary steps of tumor metastasis. Genes in *Smid's* signature also play essential roles in cell growth, proliferation, survival, angiogenesis, migration, and invasion. Tumor cells that overexpress genes in these two signatures seem to be able to invade and intravasate from primary site and disseminate through circulation. *TFF1*, *TFF3*, and *FGFR3* are present in all three categories. Genes in *Kang's* signature are mostly associated to angiogenesis, migration/invasion, EMT, and CSC factors. Many genes in this signature have been directly linked to bone metastasis of breast cancer. *MCAM*, *PTK7*, and *CTGF* are common genes in three categories. Genes in *Kang's* signature likely act toward homing, extravasation, formation of micrometastasis, and eventually metastatic colonization, in order to end this journey. Abbreviations: *EMT* epithelial to mesenchymal transition, *CSC* cancer stem cells

confirmed in patients with BC and prostate cancer [64]. Figure 2 also shows deviation toward particular functions in each signature. In *van't Veer's* signature almost two out of third number of genes (24 out of 35) are in the category of proliferation. In *Smid's* signature 13 out of 17 genes are in the category of migration. In *Kang's* signature 9 out of 16 genes are involved in functions of migration and invasion. This may show the evolutionarily pattern of tumor progression and metastasis in BC bone metastasis.

A set of well-defined genes govern key molecular pathways, and are the behind-the-scene forces of BC bone metastasis. Genes from *van't Veer's*, *Smid's*, and *Kang's* signatures control signaling pathways that have been considered as pivotal driving forces of tumor progression and metastasis. TGF-β, FGF, NFκB, WNT, PI3K, and JAK-STAT signaling pathways are induced/enhanced by genes of *van't Veer's*, and *Smid's* signatures in primary breast tumor cells. On the other hand, TGF-β, FGF, NFκB, WNT, and PI3K pathways are also induced/enhanced by genes of *Kang's signature* in bone colonized tumor cells. In both primary and distant tumors EMT program is induced by these pathways, and also several genes such as SNAI1 and MMP9 in primary tumor, and MCAM, PTK7, CXCR4, RGCC, and CTGF in distant metastatic tumors (Fig. 3) (Tables 2, 3, 4).

Smad-dependent and Smad-independent TGF-β signaling pathways are essential for EMT and BC metastases [86–88]. Smad3 and Smad4 dependent TGF-β signaling have been shown to be indispensable for the induction of EMT and metastasis [89–91]. Smad transcription factors orchestrate overexpression of several important genes involved in EMT and metastasis, including *SNAIL*, *TWIST*, and *ZEB* families of transcription factor coding genes [92]. TGF-β also participates in the activation of several key signaling pathways such as Ras/ERK, and PI3K/Akt, called Smad-independent pathways, and regulates cell growth, survival, cytoskeletal reorganization, migration, and invasion [93]. For instance, MMP9 is shown to be induced by TGF-β-induced Akt-dependent ERK pathway [94]. TGF-β stimulation leads epithelial cells to obtain mesenchymal-like features, and capability of migration, invasion, and dissemination through circulation to distant sites of metastasis, and features of stemness [95].

FGF signaling also acts as an important inducer of EMT and metastasis [96]. FGFs regulate a wide range of biological functions such as proliferation, survival, and

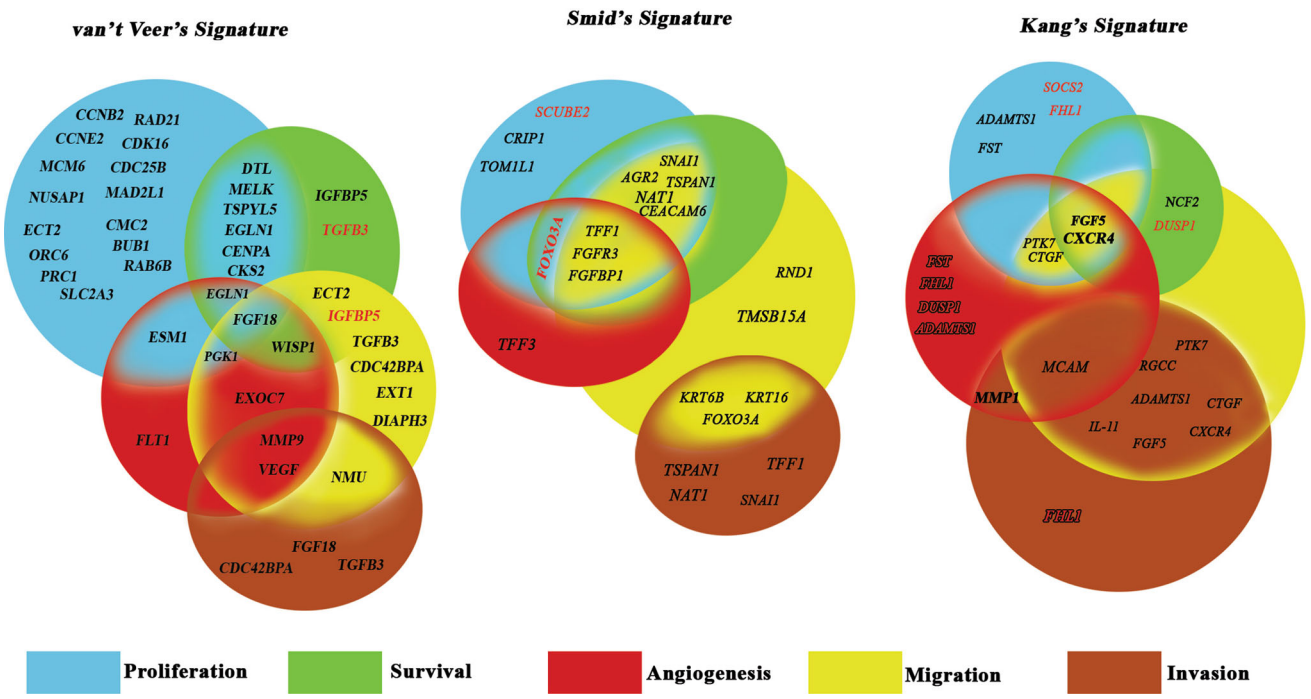

Fig. 2 Genes in *van't Veer's*, *Smid's*, and *Kang's* signatures are each deviated toward distinct functions. Certain numbers of genes in each signature are involved in certain functions categorized into: proliferation (*blue*), survival (*green*), angiogenesis (*red*), migration (*yellow*), and invasion (*brown*). Several genes have common functions, which are located in interconnected territories of circles. *Red* highlighted genes have functions against the corresponding feature (*circle*). Size of the *circles* shows the deviation toward that function in each signature. (Color figure online)

migration [97], and likely play pivotal roles in bone metastasis of BC [16]. NFκB is also an important inducer of EMT, and act through direct activation of SNAIL and ZEB family of transcription factors [98, 99]. Interestingly, it is believed that the cooperation of NFκB and TGF-β signaling pathways is critical for EMT and cancer metastasis [100, 101]. Wnt signaling is among the most important pathways involved in the induction of EMT and breast CSCs [86, 102, 103]. Notably, WNT and TGF-β signaling pathways likely induce an mutually reinforcing autocrine signaling network that is indispensable for constant expression of EMT associated transcription factors and CSC niche [87, 104]. PI3K signaling pathway is well-known for its important roles in the induction of EMT and metastasis [105]. Intriguingly, it has been shown that PI3K signaling is essential for autocrine/paracrine TGF-β associated motility, invasiveness, and metastasis [106]. JAK-STAT signaling pathway has an essential regulatory role in growth and proliferation of breast CSCs [107]. JAK-STAT signaling is associated with essential features such as survival, cell cycle regulation, self-sufficiency in growth and metastasis [108, 109]. Importantly, JAK2 also interacts and activates PI3K and RAS signaling molecules [110].

Hypoxia and hypoxia inducible factors (HIFs) seem to have pivotal roles in BC bone metastasis. HIF directly regulates several genes from *van't Veer's*, *Smid's*, and *Kang's* signatures. TFF3, EGLN1, SNAI1, MMP9, TGFB3, SLC2A3, and CTGF are of genes that are directly regulated by hypoxia. In fact, HIFs are of the essential preliminary factors that trigger gene expression programs that lead to tumor progression and metastasis, and play critical roles in the induction of EMT and stemness state in CSCs [111]. Hypoxia and HIFs are likely essential factors in the regulation of on and off states of EMT between primary and secondary tumors. In primary tumors, localized hypoxia mediates HIFs to be activated, and therefore move toward EMT/CSC induction and metastasis. At the secondary sites, however, with likely no hypoxic environment, lack of hypoxia and other factors lead to the reversion of EMT and CSC features. This phenomenon is essential for metastasis of differentiated carcinomas [87, 112] (for a comprehensive review see Ref. [112]).

It seems that a comprehensive signaling network consisting of TGF-β, FGF, NFκB, WNT, PI3K, and JAK-STAT is indispensable for breast tumor cells to progress to overt bone metastasis. Essential links between key bone metastatic factors, such as *vascular cell adhesion molecule 1* (VCAM1), *receptor activator of nuclear factor κB ligand* (RNAKL), *parathyroid-hormone related peptide* (PTHrP), and BACH1 with these pathways further confirms this

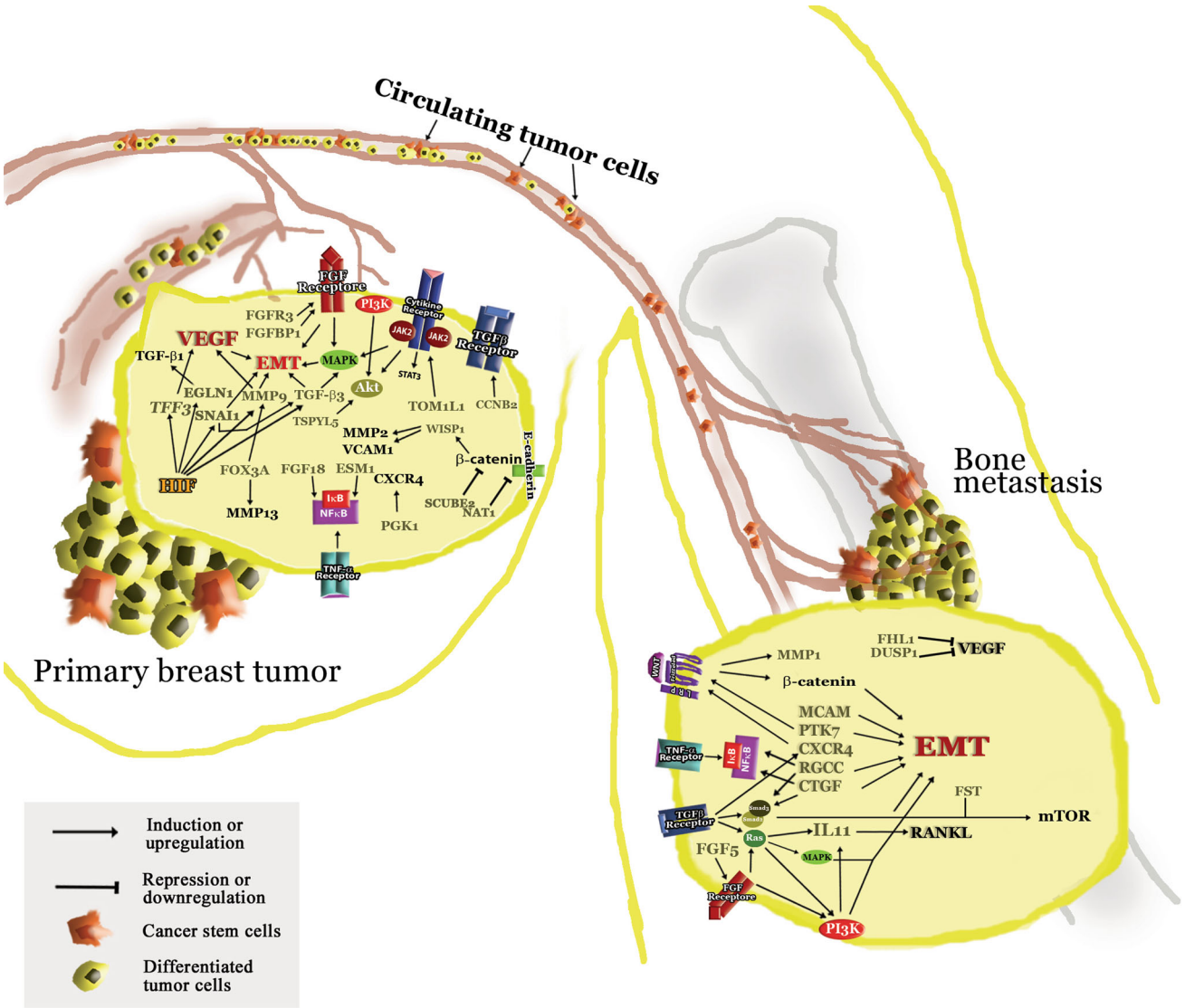

Fig. 3 Well-orchestrated genes govern key molecular pathways, and are the behind-the-scene forces of breast cancer bone metastasis. Genes in *van't Veer's*, *Smid's*, and *Kang's* signatures govern a comprehensive signaling network in primary and secondary breast tumors. TGF-β, FGF, JAK-STAT, NFκB, WNT, and PI3K pathways in primary tumor, and TGF-β, FGF, NFκB, and PI3K pathways in secondary tumor are regulated by genes in these three signatures. In the primary tumor the six signaling pathways build a comprehensive signaling network that lead toward tumor growth, proliferation, survival, angiogenesis, migration, and invasion. In primary tumor (*up-left*), genes from *van't Veer's*, *Smid's* signatures and their related signaling molecules are showed. HIF seem to have profound effects in primary tumor development and dissemination. Notably, several genes and signaling cascades induce EMT, and therefor CSC associated features. Intravasated tumor cells form the population of circulating tumor cells that disseminate, home, and extravasate into the secondary organ (bone). Importantly, the majority of differentiated circulating tumor cells (*yellow*) cannot survive the inhospitable environment while in circulation, and a small proportion of CSCs (*red*) are able to reach distant sites and form metastasis. In metastatic tumor (*down-right*), genes from *Kang's* signature lead to activation of the five pathways, which build a comprehensive signaling network that governs features like invasion, migration, EMT, and CSC formation. It is important to mention that, tumors in primary and secondary sites can be different or identical regarding their state of differentiation. In most cases secondary tumors are at the same level of differentiation as primary tumor, or even more differentiated. But in some cases, like triple negative breast cancer, both primary and secondary tumors are mostly mesenchymal, and secondary tumors are even more mesenchymal (not shown in this figure). Abbreviations: TGF-β: transforming growth factor-beta, FGF: fibroblast growth factor, JAK-STAT: Janus kinase/signal transducers and activators of transcription, NFκB: nuclear factor kappa B, WNT, and PI3K: phosphatidylinositol 3 kinase, HIF: hypoxia inducible factor, EMT: epithelial to mesenchymal transition, CSC: cancer stem cells. (Color figure online)

signaling network. VCAM1 Promotes bone metastasis by attracting and tethering osteoclast progenitors that express α4 integrin and facilitating their maturation [113, 114], and induces PI3K-Akt signaling by the mediation of Ezrin [115]. RANKL regulates bone resorption [116], migration [117], invasion [117, 118], bone metastasis [3, 117, 119,

120], and induces tumorigenesis, EMT, stemness [121], and the upregulation of MMP1 [118]. PTHrP can be induced by TGF-β [122], and activates CTGF through protein kinase A/C and ERK pathways [80]. BACH1 is a common regulator of several bone metastasis genes, including MMP1 and CXCR4 [123], induced by TGF-β [124].

Genes play the central role in development and diseases. Controlling the cellular pathways is one of the most critical duties of genes, in which reciprocal feedbacks play essential roles. Gene expression pattern in a given cell likely relates the story of a journey in which the cell is born, grow, proliferate, and/or die. Regulating the cellular behavior is the most critical tasks of gene expression machinery. Malignant behavior in cancers is tightly controlled their by gene expression pattern. Connection of gene signatures discussed in this review may provide novel insight toward better understanding the journey in which tumor cells get features of malignancy and metastasize to distant sites, and therefore providing best fit treatments for any individual cancer.

Conflict of interest The authors declare that there is no conflict of interest.

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