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Bone Metastasis: Molecular Mechanisms Implicated in Tumour Cell Dormancy in Breast and Prostate Cancer

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Abstract: Metastasis to the bone is most frequently observed in advanced cases of breast and prostate cancer. The latent development of overt metastatic lesions is associated with debilitating skeletal morbidity and eventual patient mortality. Secondary tumours in bone are derived from disseminated tumour cells (DTCs) that enter into a state of cellular dormancy. The dormant state confers resistance to conventional chemotherapeutic agents and prevents elimination of DTCs from the bone using current drug therapies. Expansion of our presently limited understanding of the molecular mechanisms underpinning disseminated breast and prostate tumour cell dormancy is critical to the future development of novel drug therapies aimed at the removal of DTCs, and thereby, the prevention of bone metastasis. This review provides an overview of the main putative molecular mechanisms underlying cellular dormancy in breast and prostate cancer bone metastasis reported from multiple experimental *in vitro* and *in vivo* models.

Keywords: Bone metastasis, breast, cancer, disseminated tumour cells, dormancy, prostate, quiescence.

INTRODUCTION

Bone metastases are a frequent complication of advanced malignancy, with breast and prostate cancer in particular having high incidence of metastasis at skeletal sites [1-3]. Infiltration of tumour cells into bone is associated with disruption of the tightly controlled process of bone remodelling, resulting in the well-established "vicious cycle of bone metastasis" that leads to increased bone resorption and, ultimately, tumour progression [4] (Fig. **1**). Once dissemination of tumour cells to the skeleton has occurred, the condition is generally considered a terminal illness [1]. In addition to this bleak prognostic outlook, a significant number of individuals with bone metastases will suffer frequent or recurrent secondary skeletal complications, described clinically as skeletal-related events (SREs), which typically include; pathological fractures, spinal-compression syndromes, bone pain and hypercalcaemia [5]. The development of cancer-related skeletal morbidity is associated with considerable personal, social and financial burden, and a markedly diminished quality of life [6]. The prevention of metastatic bone disease is therefore of primary importance to contemporary clinical practice.

The current therapeutic standard for treatment of cancer-related bone disease is that of bone-targeting anti-resorptive agents, including the bisphosphonates and the fully humanised, synthetic monoclonal antibody denosumab [1, 7]. These agents are aimed at reducing the rate of disease progression, lengthening survival, and decreasing the incidence of SREs. Their relative successes in doing so has led to metastatic bone disease now being considered a chronic condition in many cases [8]. However, long-term mortality rates remain largely unchanged due to the latent development of secondary tumours [9]. Secondary metastatic lesions are invariably derived from disseminated tumour cells (DTCs) that have entered into a reversible state of cellular dormancy or proliferative arrest, making them inherently unresponsive to conventional anti-mitotic therapies. A number of years or even decades following initial treatment, these cells may emerge from quiescence to form secondary tumours that lead to skeletal complications and eventual patient mortality [10].

At present, it remains virtually impossible to isolate DTCs from clinical samples in the earliest stages of metastasis. This is primarily because the systemic spread of tumour cells to the bone occurs as early as the pre-invasive stages of disease, when tumours are either asymptomatic or clinically undetectable [11, 12]. While it is more feasible to study patient-derived DTCs in the later stages of disease, their isolation is technically difficult and bone marrow biopsy itself is associated with some risk of procedural complications. In addition, the supply of clinical samples is often problematic due to a lack of willing compliance on the part of both patients and their clinicians [13]. As a consequence, the vast majority of the current understanding of cellular dormancy has been extracted using pre-clinical *in vitro* and *in vivo* models. Unfortunately however, the precise biological mechanisms underpinning the induction and regulation of the quiescent state still remain to be identified. Given the restrictions surrounding the use of patient-derived DTCs, further advances in the knowledge of tumour cell dormancy in bone will predominantly rely on the continued development and usage of reliable pre-clinical model systems in which the molecular mechanisms that cause cellular dormancy can be successfully unravelled. The

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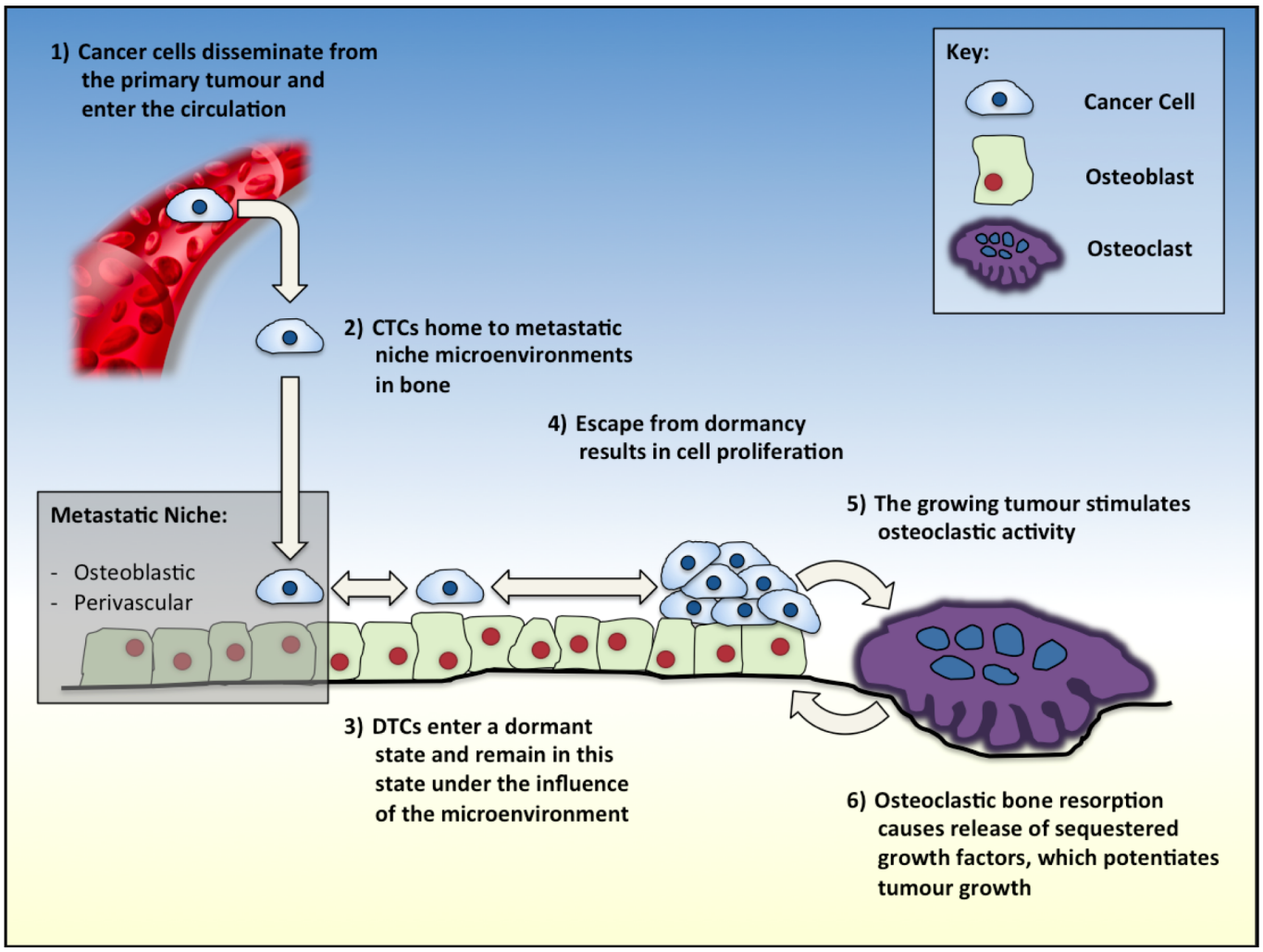

Fig. (1). Disease Pathogenesis in Bone Metastasis. Cells from the primary tumour are shed into circulation. Circulating tumour cells (CTCs) that preferentially metastasise to bone are suggested to exploit the same physiological mechanisms that instruct homing of haematopoietic stem cells (HSCs). Disseminated tumour cells occupy niches within bone that are suggested to be composed of various cell types, including bone marrow stromal cells, osteoblasts, vascular endothelial cells and immune cells. Niche cells interact with tumour cells *via* integrin and chemokine axes, such as α4β1-vascular cell adhesion molecule-1 (VCAM-1) and CXCL12-CXCR4, respectively. Within the bone, tumour cells are maintained in a growth-arrested state that is regulated by signals encoded within the bone marrow microenvironment. In some cases, escape from dormancy is triggered, resulting in metastatic progression, formation of a secondary tumour in bone, and further dissemination to extra-skeletal sites in some cases. Growing tumour cells secrete molecules that stimulate formation, activation and maturation of osteoclasts increasing bone resorption, including IL-1, IL-6, parathyroid hormone-related protein (PTHrP) and tumour necrosis factor alpha (TNF-α). In turn, resorptive activity releases a number of growth factors sequestered in the bone matrix, such as transforming growth factor beta (TGF-β) and insulin-like growth factor 1 (IGF-1), which further stimulate tumour growth.

further elucidation of dormancy-mediating pathways in this way is likely to be pivotal to the development of effective therapeutic strategies for targeting dormant DTCs and considerably enhancing the long-term prognosis of patients with skeletal metastasis.

INDUCTION OF CELLULAR DORMANCY

The modulation of stress tolerance pathways, reduced mitogenic signalling, and induction of autophagy have emerged as central mechanisms in the initiation and subsequent regulation of dormancy (Table **1**). In particular, the balance between the extracellular signal-regulated kinase (ERK) and p38 pathways, and the disruption of phosphatidylinositide 3-kinase (PI3K)-protein kinase B (PKB) signalling are implicated as key mediators [14, 15]. These processes are seemingly initiated during systemic dissemination or may occur later in response to the growth-restrictive metastatic niche microenvironment encountered within the bone.

Loss of ECM Signal Transduction

In a transgenic mouse model of breast cancer, White *et al.* [16] reported that the loss of β1-integrin from tumour cells was linked with resistance to apoptotic cell death and entry into a growth-arrested quiescent state. Earlier studies undertaken in human epidermoid carcinoma models *in vitro*

Table 1. Dormancy cues and putative markers in breast and prostate cancer.

Dormancy Inductive Process	Markers		References
Stress-Signalling	Down-regulated:	ERK	[14, 17-20]
		FAK	[17-19, 33]
		PKB	[15, 24]
		Cyclin D1	[15]
		CDK	[15]
	Up-regulated:	p38	[14, 16-20, 33]
		PERK	[33, 38]
		BHLHE41	[20]
		p53	[20]
		p27	[15]
		GRP-78	[25, 33]
		GRP-94	[25]
		PDI	[25]
Autophagy	Down-regulated:	mTORC1	[36-37]
		RhoA	[40, 41]
	Up-regulated:	ULK-1	[36, 37]
		ATG-5	[40, 41]
		ATG-7	[40, 41]
		ATG-12	[40, 41]
		ATG-13	[37, 40, 41]
		MAP1LC3B-I/II	[40, 41]
		RhoC	[40, 41]
		AMPK	[38]
		JNK	[40, 41]

AMPK = AMP-activated protein kinase; ATG = autophagy-related protein; BHLHE41 = basic helix-loop-helix family member e41; CDK = cyclin-dependant kinase; ERK = extracellular signal-regulated kinase; FAK = focal adhesion kinase; GRP = glucose-regulated protein; MAP1LC3B = microtubule-associated protein 1A/1B light chain 3B; mTORC1 = mTOR complex-1; PDI = protein disulphide isomerase; PERK = p38-PRKR-like endoplasmic reticulum kinase; PKB = protein kinase B; Rho = ras-homologue gene family member; ULK = unc-51-like kinase.

and *in vivo* demonstrated that induction of dormancy followed abolition of fibronectin-dependent proliferative signalling *via* the FAK-Ras-ERK pathway in response to reduced urokinase receptor (uPAR)-mediated α5β1-integrin activation [17-19]. In all of these studies, disruption of β1-integrin function was seen to occur in conjunction with the sustained over-activation of the p38 mitogen-activated protein kinase (MAPK) pathway.

Using a phosphorylation-linked green fluorescent protein (GFP) reporter system, Aguirre-Ghiso *et al.* [14] later demonstrated that deactivated ERK signalling in conjunction with high p38 activity is conducive to a protracted state of dormancy, while the opposite (high ERK:p38 ratio) is a prerequisite for mitogenesis. More recently, this pattern of p38 and ERK signalling has been associated with enhanced expression of tumour suppressor proteins BHLHE41 and p53 leading to dormancy in head and neck squamous cell carcinoma (HNSCC) models [20]. The relevance of these mechanisms in relation to the findings of White *et al.* [16], and to dormancy following bone metastasis in breast cancer remains to be fully elucidated.

The dysregulation of integrin expression during malignant transformation in breast cancer is well documented, and is suggested to occur during epithelial-mesenchymal transition (EMT) and acquisition of the migratory phenotype [21]. These processes facilitate the disruption of cellular adhesion to the extracellular matrix (ECM), and are regarded as being prerequisite for systemic dissemination [22]. However, the critical importance of ECM-adhesion to normal cell cycle progression is widely accepted, and numerous studies have demonstrated the dependence of growth factor driven cell-cycle progression on integrin-mediated anchorage-dependent signal transduction in breast tissue [23]. The findings of White *et al.* [16] might, therefore, be seen to indicate that the loss of ECM-anchorage and transduction of microenvironmental signals from within the primary tumour results in a protective state of dormancy as a survival mechanism in cells which undergo integrin-loss prior to systemic dissemination.

Evidence from a more recent investigation by Humtsoe and Kramer [15] in squamous cell carcinoma models appears to support this hypothesis. In this study, cells within non-adherent multicellular aggregates were able to circumvent the loss of anchorage-dependent signals through autophosphorylation of the epidermal growth factor receptor (EGFR). This phenomenon uncoupled ERK activation from PI3K-PKB signalling due to ineffective GRB2-associated-binding protein-1(Gab-1) recruitment. Overall, this led to enhanced p27 expression but no concomitant rise in cyclin D1; cells were therefore able to survive but unable to proliferate, instead entering into a protective state of quiescence. Direct experimental evidence for the role of

these pathways in cellular dormancy in breast cancer does remain outstanding. However, clinical samples of DTCs from a small cohort of patients with advanced disease showed markedly reduced PKB activation compared to primary tumour cells in one study, indicating that they might be relevant [24].

The Unfolded-protein Response

Following systemic spread and arrival at metastatic sites, DTCs may encounter growth-restrictive microenvironmental conditions, including hypoxia, hypoglycaemia and acidosis [25]. Cellular oxidative and nutritional stress affects the fidelity of post-translational protein folding and assembly within the endoplasmic reticulum (ER) lumen. This initiates a number of down-stream signal transduction pathways that act to maintain cellular homeostasis. This adaptive stress tolerance mechanism is termed the unfolded-protein response (UPR) [26]. Studies in HNSCC models have demonstrated that ER-stress induced by nutrient deprivation resulted in enhanced p38 activity and induction of the ER-stress marker GRP-78, and was associated with entry of cells into dormancy and resultant prolonged survival [27]. In addition, Bartkowiak *et al.* [25] demonstrated significantly elevated expression of ER-stress-related proteins GRP-78, GRP-94 and protein disulphide isomerase (PDI) in a novel cell lineage derived from DTCs isolated from the bone marrow of a breast cancer patient with no clinical signs of overt metastases. It has been supposed that this induction of ER-stress chaperones might have occurred in response to hypoxia, hypoglycaemia and the resultant reduction in cellular adenosine 5′-triphosphate (ATP) levels encountered by DTCs within the bone marrow [10]. Hence, the UPR may function, at least in part, to promote the entry into dormancy and survival of dormant DTCs in response to the hostile microenvironmental conditions encountered following systemic dissemination.

The UPR has also been implicated in mediating the pro-dormancy effects associated with expression of cytokeratin-19 (CK-19). The up-regulation of CK-19 in metastatic breast cancer is well documented, and CK-19 expression is considered to be an independent prognostic indicator in breast cancer and a variety of other metastatic cancers [28-31]. In addition, CK-19 has long been used as a biomarker for the detection of CK-19-positive DTC sub-populations in circulation and in the bone marrow [32]. Fairly recently, Bambang *et al.* [33] demonstrated that expression of CK-19 in the BT549 human breast cancer cell lineage appeared to result in enhanced p38-PRKR-like endoplasmic reticulum kinase (PERK) signalling, increased levels of GRP-78, and down-regulated FAK. Increased p38 signalling appeared to negatively regulate ER protein-29 (ERp29) expression, most probably mediated *via* MAPK and the transcription factor known as X-box binding protein-1 (XBP-1), leading to compromised processing of luminal secretory protein within the ER. These effects were causally associated with cell cycle arrest and a markedly reduced susceptibility to the cytotoxic effects of chemotherapeutic agents doxorubicin and cisplatin. These results suggest that CK-19 expression by a sub-population of metastatic breast tumour cells contributes to their entry into dormancy, leading to enhanced survival and chemotherapeutic resistance. It may be that these cells are better adapted to survive chemotherapeutic treatment and the dissemination process, and thus go on to metastasise to the bone where they may remain dormant or progress to form secondary metastatic foci.

The Role of Autophagy

Autophagy, first described by Ashford and Porter [34], is a tightly controlled catabolic process that involves lysosomal sequestration and degradation of cytoplasmic organelles or cytosolic constituents [35]. Under normal physiological conditions, autophagic induction is primarily regulated by the suppressive action of mTOR complex-1 (mTORC1), a master regulatory complex formed within cells between the mammalian target of rapamycin (mTOR) and regulatory-associated protein of mTOR (Raptor), on unc-51-like kinase-1 (ULK-1) and autophagy-related protein-13 (ATG-13). During periods of cellular stress or nutrient-deprivation, the mTORC1 kinase activity is halted and the subsequent activity of ULK-1 and ATG-13 is able to initiate autophagic degradation of cellular material, thus facilitating the recycling of metabolites and energy [36, 37]. In this capacity, autophagy may function as an adaptive mechanism to maintain metabolic fitness and facilitate cellular quiescence in response to adverse conditions encountered following systemic dissemination of cancer cells. A number of studies have implicated autophagy in mediating cell survival following loss of ECM-attachment dependent signalling, as is experienced during the dissemination process.

In an *in vitro* model, MCF-10A human breast epithelial cells grown in suspension for a 12-hour period showed markedly enhanced PERK signalling that caused activation of AMP-activated protein kinase (AMPK). Suspension-induced AMPK signalling was shown to result in deactivation of mTORC1-mediated signalling and induced an autophagic response that seemingly protected cells from ECM-detachment-induced death by anoikis. A similar response was also observed when β1-integrin-mediated cell adhesion was blocked with the function-blocking antibody AIIB2 [38]. These findings indicate a potential mechanistic explanation for the enhanced autophagosome formation in response to β1-integrin blockade noted in earlier studies undertaken by Fung *et al.* [39] using a similar MCF-10A model. This also suggests that the dormant state into which β1-integrin-deficient cells entered in studies undertaken by White *et al.* [16] could, at least in part, be mediated by autophagic mechanisms.

Autophagic induction may also function to induce a protective state of dormancy that contributes to a conferred ability of DTCs to withstand targeted therapies. Treatment of MCF-7 and MDA-MB-231 human breast cancer cells with a number of farnesyl-transferase inhibitors (FTIs), a novel class of Ras-activity-modulating experimental anti-cancer agents, resulted in a reversible state of dormancy [40, 41]. In both instances, entry into the dormant state was seemingly mediated by concomitant hypoactivation of Ras-homologue gene family member A (RhoA), hyperactivation of Ras-homologue gene family member C (RhoC), and upregulation of c-jun N-terminal kinase (JNK). Diminished RhoA activation was noted as being consistent with the established

in vitro model of breast cancer dormancy of Barrios and Wieder [42]. In the MCF-7 model, a number of proteomic changes indicative of autophagy were detected. Specifically, the enhanced expression of ATG-7 and ATG-13 was observed as an early event, and detectable levels of ATG-12, ATG-5 and MAP1LC3B-I/II were present for a prolonged period of time following FTI exposure. Concomitant exposure of cells to FTIs and 3-methyladenine, a specific inhibitor of the macroautophagic pathway, resulted in a marked reduction in the number of dormant cells, strongly implying that autophagy was conducive to dormancy in response to FTI treatment [41].

It is possible that similar autophagic pathways mediate DTC resistance to endogenous pro-apoptotic factors. In particular, autophagy might be induced as a survival mechanism in response to cell death signalling by the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) which is expressed abundantly, as both membrane-bound and soluble forms, within the bone marrow microenvironment [43]. In support of this hypothesis, autophagic maintenance of cell viability in the presence of TRAIL has been documented previously *in vitro* using both leukaemia and colorectal carcinoma cell lines [44]. Given the long-term persistence of DTCs following breast and prostate metastasis to bone, it seems feasible that TRAIL-induced autophagic mechanisms might contribute to the cellular dormancy that is observed clinically in these cancer types, although this remains to be established.

MICROENVIRONMENTAL REGULATION OF THE DORMANT STATE

Tumour cells that survive metastatic dissemination home to niche microenvironments within distant tissues [45]. While in many cancer types there is a relatively predictable pattern of secondary disease, DTCs can often be found in a non-proliferative state in a number of target organs, including the bones, liver and lungs [20, 45]. This phenomenon implies a disparity in the capacity of different tissues to support DTC growth, and supports the established "seed and soil' theory of metastasis originally proposed by Stephen Paget over a century ago (reviewed by Langley and Fidler [46]). The contemporary conceptualisation of Paget's theory proposes that it is the compatibility or interactions between the cellular phenotype of DTCs (the seeds) and the signals encoded within specific niche microenvironments (the soil) that is the primary determinant of whether DTCs are able to proliferate and produce overt secondary lesions, or are forced to enter a protracted state of mitotic arrest [47]. Therapeutic targeting of the microenvironment might thus enable disruption of pro-dormancy signalling, causing DTCs to emergence from quiescence, and allowing their removal using conventional anti-mitotic agents.

GAS6 Receptor Signalling

In vivo model studies have demonstrated that disseminated prostate cancer cells (PCCs) preferentially migrate to osteoblast-rich regions of bone and are shown to directly compete for occupancy of the haematopoietic stem cell (HSC) niche [48, 49]. Molecular pathways which are known to be critical to HSC niche selection, such as the annexin II and chemokine receptor-4 (CXCR4)/chemokine ligand-12 (CXCL12) axes, have been centrally implicated in this process [49, 50]. Exploration of the role of osteoblastic annexin-II in directing interactions between disseminated PCCs and the HSC niche showed that it significantly enhanced PCC expression of the AXL subfamily of receptor-tyrosine kinases [51]. This group of receptors, which includes AXL, Tyro3 and MER, selectively bind the stromal-derived growth arrest-specific 6 (GAS6) growth factor [52]. Enhanced expression of AXL receptors and consequent ligation by GAS6, mediated by annexin-II, was shown to increase survival, confer resistance to chemotherapy and reduce the mitotic activity of PCCs by inducing G_0 arrest [51].

In PC-3 and DU-145 prostate cancer subcutaneous xenograft models, DTCs isolated from bone marrow demonstrated markedly reduced proliferative activity during fluorescence-activated cell sorting (FACS) analysis of Ki67 when compared with cells of the primary tumour [53]. Primary tumour cells possessed a significantly reduced AXL:Tyro3 expression ratio compared to cells grown *in vitro*, whereas DTCs isolated from tumour-free marrow showed increased expression of AXL and down-regulated Tyro3 compared with cells taken from both primary tumours and from secondary metastatic foci. Evidence from a study undertaken by Mishra *et al.* [54] indicates that such alterations in receptor sub-type profiles might be partially dependent on stabilisation of AXL expression regulated by hypoxia-inducible factor 1-alpha (HIF1-α). Irrespective of the mechanism, the observations of Taichman *et al.* [53] seemingly demonstrate that differential expression of AXL receptor sub-types is a key determinant of the tightly controlled switch between proliferative and dormant phenotypes in prostate cancer bone metastasis. More specifically, a low AXL:Tyro3 receptor ratio appears to favour cellular proliferation while a high AXL:Tyro3 ratio is seemingly conducive to a quiescent state. Such induction of a dormancy-permissive phenotype during niche selection closely resembles the situation that is seen during HSC occupancy of osteoblastic niche within the bone marrow [55, 56]. Hence, it may be that other microenvironmental mechanisms known to instruct HSC function are also responsible for regulation of dormancy in DTCs resident within the bone marrow.

TBK-1 Expression

Recent evidence reported in a study by Kim *et al.* [57] indicates that cell-niche interactions that regulate prostate cancer cell dormancy within the bone microenvironment may be mediated by modulation of mTOR signalling. Kim *et al.* [57] found that binding of PCCs to bone marrow stromal cells (ST2 lineage) during *in vitro* co-culture markedly increased the number of cells in proliferative arrest. This sub-population of cells, isolated based on Ki67 expression by FACS, were also noted as having significant chemoresistance to the anti-mitotic agent docetaxel. In both PC-3 and C4-2B prostate cancer cell lineages, real-time quantitative polymerase chain reaction (qPCR) analyses revealed hyper-expression of the serine/threonine-protein kinase TBK1 (TANK-binding kinase-1) in response to ST2-cell binding interactions.

In cancer, TBK1 has been implicated in mediating a number of functions that facilitate both malignant transformation and cell survival [58-60]. In the study undertaken by Kim *et al.* [57], immunofluorescent staining

of PCCs taken from their *in vitro* co-culture model revealed co-localisation of TBK1 with mTOR around the nuclear periphery. Rapamycin-induced inhibition of mTOR increased the proportion of dormant PCCs ($Ki67^-$) and enhanced resistance to an array of conventional chemotherapeutic agents in both PC-3 and C4-2B cell lines. Lentiviral shRNA knockdown of TBK1 resulted in a significant increase in mTOR activity and loss of chemoresistance. In addition, in stem cell-like ($CD133^+/CD44^+$) sub-populations of PC-3 and C4-2B cells isolated from mouse bone marrow, TBK1 levels were significantly increased. *In vitro*, shRNA-induced loss of TBK1 not only caused enhanced mTOR activation but also was associated with a marked decrease in the $CD133^+/CD44^+$cells. This effect was significantly reduced in the presence of rapamycin. When taken together, these studies demonstrate that an inverse regulatory relationship exists between TBK1 and mTOR, and it appears that PCC-stromal interactions that increase TBK1-induced inhibition of mTOR are seemingly important in the formation of stem cell-like sub-populations, maintenance of dormancy, and chemoresistance in prostate cancer. However, the mechanisms by which PCC-niche interactions induce enhanced TBK1 expression, and by which TBK1-mediated inhibition of mTOR leads to the observed chemoresistance and proliferative arrest remain to be elucidated.

A study in quiescent ovarian tumours, undertaken by Lu *et al.* [61], showed that dormancy was mediated by autophagic induction due to enhanced expression of the Ras homologue AHRI and resultant inhibition of the PI3K-PKB-mTOR pathway. The dramatic reduction in tumour regrowth in the presence of doxorubicin following chloroquine-induced inhibition of autophagy also indicated that autophagy is likely to be an important survival mechanism enabling dormant tumour cells to survive chemotherapy. In light of this, it is possible that TBK1-induced mTOR inhibition results in autophagy, and that this is responsible for the dormancy and enhanced chemoresistance in prostate cancer cells reported by Kim *et al.* [57].

BMPR-2 Signalling

While attempting to further elucidate bone stroma-derived secretory factors that may have modulatory effects on DTC dormancy in bone, Kobayashi *et al.* [62] noted that the conditioned medium from normal bone marrow stromal cells (HS5 lineage) was able to significantly reduce prostate cancer cell proliferation but did not elicit any cytotoxic effects. On further investigation, reduced proliferative activity appeared to strongly correlate with markedly enhanced p38-MAPK signalling, loss of ERK signalling, and simultaneous induction of the N-myc downstream-regulated gene 1 (NDRG1) metastasis suppressor protein, and the cyclin-dependent kinase (CDK) inhibitors p21 and p27. Addition of small molecule inhibitors that block the action of secretory factors produced by bone stromal cells showed that inhibition of bone morphogenetic protein (BMP) signalling re-establish the dormancy-associated signalling profile observed in the presence of HS5 conditioned medium. Analysis of the effects of purified BMPs *in vitro* identified only BMP7 as being able to activate p38, NDRG1, p21 and p27, and diminish ERK signalling, eliciting a state of dormancy. This dormant state was reversed following removal of BMP7 or knockdown of its receptor, bone morphogenetic protein receptor type II (BMPR2).

The increased expression of NDRG1, an apparent effector in BMP7-mediated dormancy, has previously been shown to inversely correlate with occurrence of bone metastasis in prostate cancer patients [63]. NDRG1 activity has also been associated with suppression of metastatic activity in cancers of the breast and colon [64, 65]. NDRG1 apparently achieves these effects *via* disruptive interactions with low-density lipoprotein receptor-related protein 6 (LRP6), the specific receptor of the Wnt ligand [66]. When taken together, these studies suggest that the BMP7-BMPR2-NDRG1 axis plays a pivotal role in maintaining a state of cellular dormancy that is likely to contribute to the suppression of metastasis in prostate cancer, and probably other cancer types, and that loss of BMPR2 signal transduction may precede metastatic progression.

TGF-β2 Signalling

Transforming growth factor-beta 2 (TGF-β2) is a cytokine known to be present within the bone marrow where it has been shown to regulate haematopoietic progenitor cell development [67]. Recently, Bragado *et al.* [68] demonstrated that TGF-β2 signals present in the bone marrow are able to maintain HNSCC DTCs in a dormant state. In this study, GFP-tagged dormant HEp-3 cells isolated from the bone marrow of mice showed a prolonged 87% reduction in proliferative capacity compared to dormant cells isolated from lung. The bone marrow microenvironment thus appeared to specifically instruct activation of persistent dormancy-inductive signalling programmes within DTCs. Expression profile analyses revealed up-regulation of TGF-β2 in dormant cells from bone at greater than five-times the level present in either the parental cell line or DTCs isolated from the lungs. TGF-β2 levels were also significantly elevated in bone marrow compared to lung tissue and the basal culture medium used *in vitro*.

Within dormant DTCs, p38α/β was strongly activated while ERK signalling was diminished. The resultant high p38:ERK signalling ratio led to induction of metastatic suppressor proteins BHLHE41 (also known as DEC2 or SHARP1) and p27, and subsequent repression of CDK4, leading to a state of cellular quiescence. This dormancy-instructive phenotype was specifically restored in the presence of the β2-isoform of TGF only. However, TGF-β2-mediated dormancy was critically dependent on the co-expression of TGF-β-receptor-I (TGFβRI) and TGFβ-receptor-III (TGFβRIII) for the down-stream activation of SMAD 1 or SMAD 5 that is necessary for subsequent induction of p27 and mitotic arrest. Inhibition of TGFβRI or p38α/β favoured escape from dormancy and increased overall metastatic burden [68]. Similar findings were reported by Marlow *et al.* [69] following TGFβRI or p38-MAPK inhibition in growth-arrested breast cancer cell lines (MCF-7 and SUM159) generated within a 3D *in vitro* model of the dormancy-permissive bone marrow niche. This finding, therefore, strongly suggests that TGF-β2-mediated mechanisms elucidated by Bragado *et al.* [68] may be of equal importance to dormancy in breast cancer following metastasis to bone.

NK1R-Tr Status

Tachykinin family proteins are produced by alternate exon splicing of the *Tac1* gene followed by post-translational modification, and are constitutively expressed by breast cancer and bone marrow stromal cells [70, 71]. These molecules exert their biological effects through ligation of three different G protein-coupled transmembrane receptors; NK1R, NK2R and NK3R [71]. In both primary and metastatic breast cancer, interaction of tachykinins with the truncated variant of NKR1 (NK1R-Tr) expressed by malignant cells has been shown to promote mitogenesis. In addition, reduced expression of the full-length NKR1 variant (NKR1-FL) and overexpression of NK1R-Tr has been associated with a propensity for malignant transformation, as well as an enhanced capacity for invasiveness and metastatic activity [71, 72]. In a recent study conducted by Zhou *et al.* [73], knockdown of NKR1-Tr using shRNA in the MDA-MB-231 cell line resulted in a markedly reduced growth rate compared to cells of the parental lineage. Co-culture of MDA-MB-231 cells with bone marrow-derived human mesenchymal stem cells (BM-HMSCs) resulted in reduced NK1R-Tr expression and concomitant cell-cycle arrest. This observation was linked to markedly increased levels of CXCL12 in the conditioned medium taken from the co-culture system which were not present in that harvested from MDA-MB-231 cells grown alone. When taken together, these results imply that reversion to a low NK1R-Tr phenotype in metastatic breast cancer is involved in regulation of DTC dormancy in bone, and that BM-HMSC-derived CXCL12 instructing reduced NK1R-Tr expression by breast tumour cells might mediate this process. However, both a detailed mechanism and evidence for the relevance of this purported dormancy mechanism to breast cancer bone metastasis *in vivo* remain to be established.

bFGF Signalling

In contrast to their suggested roles in induction of breast cancer cell dormancy, $\alpha5\beta1$ integrin expression and re-acquisition of an activated PI3K-PKB signalling axis have been previously implicated in maintenance of breast cancer dormancy in the bone marrow. Basic fibroblast growth factor (bFGF) is a well-known mammary differentiation factor that is present in the bone marrow and is capable of modulating integrin expression. Korah *et al.* [74] first reported that bFGF was able to induce the reversible up-regulation of integrin $\alpha5\beta1$ lost during malignant transformation. Reconstitution of $\alpha5\beta1$ integin in this investigation was associated with fibronectin-ligation-dependent survival through inhibition of apoptosis that appeared to be mediated by enhanced PI3K-PKB signalling. In a subsequent investigation by Barrios and Wieder [42], PI3K pathway activation was shown to occur in a bFGF-dependent manner independent of $\alpha5\beta1$ activation. In this case, the mitotic arrest necessary for maintained quiescence was shown to occur through the stabilisation of cortical F-actin fibres following inactivation of RhoA. This loss of RhoA activity was preceded by PI3K-dependent activation of GTPase regulator associated with focal adhesion kinase (GRAF), a RhoA GTPase-activating protein-like complex, and the subsequent $\alpha5\beta1$ integrin-dependent recruitment of FAK. Hence, dormancy of disseminated breast cancer cells within fibronectin-rich compartments of the bone marrow may be initiated and maintained, at least in part, through simultaneous bFGF-dependent signalling *via* PI3K and $\alpha5\beta1$.

Thrombospondin-1

The multi-functional endothelium-derived matrix glycoprotein thrombospondin-1 (TSP-1) has previously been implicated in regulating both micrometastatic and cellular dormancy, suggested to occur through negative regulation of angiogenesis [75, 76]. Recently, Ghajar *et al.* [77] showed that TSP-1 surrounding the mature vascular endothelium acts as an angiocrine tumour suppressor that is able to maintain breast tumour cells (HMT-3522 T4-2, MCF-7 and MDA-MB-231 lineages) in a protracted dormant state within the murine bone marrow *in vivo*. This finding was confirmed using an organotypic model of the human bone microvascular niche *in vitro*. In a similar model of the developing vasculature, where TSP-1 expression was significantly diminished, the dormant cellular phenotype was not recapitulated.

This study supports the hypothesis that endothelial cells and secretory factors deposited within their surrounding basal lamina comprise, at least in part, a dormancy-permissive perivascular niche. It also appears to demonstrate vascular regulation of tumour cell behaviour, a drastic shift from the long-established existing paradigm, and also suggests the potential existence of other, as yet uncharacterised, vascular regulators of cellular dormancy. In addition, while the anti-angiogenic activity and effects of TSP-1 on endothelial cells are well characterised (reviewed by Lawler and Lawler [78]), the molecular mechanism by which it is apparently able to regulate cellular dormancy remains to be elucidated.

Gap Junctional Intercellular Communications and MicroRNAs

Recently, Park *et al.* [79] reported that low levels of CXCL12 were able to enhance gap junctional intercellular communication (GJIC) between confluent breast cancer cells (MDA-MB-231) by increasing the translation and protein kinase-C-mediated activation of the connexin protein Cx43.In a previous study, gap junctional transmission of quiescence-inducing microRNA (miRNA) molecules miR-127, -197, -222, and -223 from primary bone marrow-derived stromal cells to cells of the MDA-MB-231 and T47D breast cancer lineages was linked to induction of dormancy during co-culture [80]. Transmission of these miRNAs resulted in a marked decrease in cyclins D1, D3, C and CDK4, and a concomitant increase in p21, leading to proliferative arrest in the G_0 phase of the cell cycle.

In their study, Lim *et al.* [80] also noted that miRNA transfer *via* stromal-derived exosomes was linked to tumour cell quiescence, although to a lesser degree than that occurring *via* GJIC. However, the exosomal transfer of miRNA leading to dormancy is concurrent with a more recent investigation undertaken by Ono *et al.* [81]. In this study, bone marrow-metastatic MDA-MB-231 variant cells acquired a quiescent phenotype in co-culture with BM-HMSCs, in the presence of conditioned medium from BM-HMSCs, or following uptake of exosomes derived from the BM-HMSC cells. Amongst 44 miRNAs that were found to

be present in BM-HMSC-derived exosomes at an elevated level, miR-23b was identified as being able to reconstitute the dormant phenotype observed in earlier experiments. Analysis of several putative target genes identified the MARCKS gene, which encodes myristoylated alanine-rich C kinase substrate, as the target of miR-23b action. Analysis of patient bone marrow aspirates demonstrated the coexistence of BM-HMSCs amongst disseminated breast tumour cells, and that these DTCs had elevated levels of miR-23b and diminished MARCKS expression compared to primary breast tumour tissue, thus corroborating the experimental data. The combined findings of these studies not only identify two means by which bone marrow-derived miRNAs reach DTCs resident within the bone microenvironment, but also demonstrate that these miRNAs appear to play a role in the promotion of DTC dormancy within the metastatic niche. This finding also suggests that there are potentially several other miRNAs that might play important roles in the induction and maintenance of DTC dormancy following metastasis to bone but, as yet, these remain unidentified.

OTHER MOLECULES IMPLICATED IN CELLULAR DORMANCY

Exogenous Erythropoietin

Erythropoietin (Epo) primarily functions to enhance survival, stimulate growth and directing the differentiation of erythroid precursors within the bone marrow during haematopoiesis [82]. Epo has long been used, along with other erythropoiesis-stimulating agents (ESA), in the treatment of cancer-related anaemia [83]. A number of more recent meta-analyses have, however, revealed a possible association between ESA administration and worsened patient prognosis [84-86].

Following their demonstration of preferential homing of prostate cancer to the HSC niche in bone, Shiozawa *et al.* [87] proposed that these observations might have been due to Epo-mediated expansion of the number of HSC niches resulting in enhanced potential for metastatic invasion. Their findings indicated that Epo did not appear to expand niche number or size, enhance metastasis, or stimulate prostate cancer cell growth. However, Epo did confer protection to apoptosis induced by serum deprivation *in vitro* and following ablation of the protective HSC niche by ganciclovir treatment in murine models *in vivo*. Similarly, Todaro *et al.* [88] have described pro-survival effects of Epo. In this study, stem-like breast cancer cells isolated from surgical biopsies appeared to acquire resistance to the chemotherapeutic agents doxorubicin, taxol and 5-fluorouracil in the presence of Epo. These protective effects appeared to be mediated by an up-regulation of the stress-related pro-survival protein Bcl-xL and activated PKB, the latter of which has previously been associated with bFGF-mediated maintenance of breast cancer cell dormancy in bone [42, 74]. It is possible, therefore, that these mechanisms are responsible for the anti-apoptotic effects of Epo reported by Shiozawa *et al.* [87], and may contribute to maintaining DTCs in a protective state within the bone marrow microenvironment. An increased number of dormant DTCs within bone enhances the potential risk that one will be triggered to emerge from dormancy and go on to form a metastatic lesion. It may be through encouraging persistence of DTCs in the bone marrow in this manner that ESA therapy negatively impacts long-term patient survival.

Autocrine sPDZD2

PDZD2 (PDZ-containing protein-2) is a 301kDa intracellular protein with six-PDZ domains that localises to the endoplasmic reticulum and nuclear periphery. PDZD2 is post-translationally cleaved, in a caspase-3-dependent manner, to yield the 37kDa secreted PDZ domain-containing fragment, known as sPDZD2 [89, 90]. While very little is presently known about the biological activity or function of PDZD2 proteins, the autocrine secretion of sPDZD2 has been implicated in the induction of cellular dormancy programmes in both prostate and breast cancer.

Chaib *et al.* [91] first described the increased expression of full-length PDZD2 in prostate cancer. In subsequent studies undertaken by Tam *et al.* [89] and Tam *et al.* [92], endogenous expression of PDZD2 and its secreted cleavage product were detectable in the lysates and conditioned medium collected from a number of breast and prostate cancer cell lines. Postulating that autocrine secretion of sPDZD2 might have a functional role in prostate cancer pathogenesis, Tam *et al.* [89] demonstrated that recombinant sPDZD2 was able to elicit a concentration-dependent inhibition of cell proliferation in PC-3, DU-145 and 22Rv1 human prostate cancer cell lines. In a later study, sPDZD2 was observed to exert similar concentration-dependent effects on the proliferation of wild-type p53-positive MCF-7 human breast cancer cells [92].

In the presence of sPDZD2, up-regulated p21 and p53 were detected in conjunction with β-galactosidase expression in S-phase-arrested DU-145 cells. In the MCF-7 breast cancer model, growth arrested cells did not appear to express senescence markers, and while p53 expression was increased, p21 levels remained unaltered. Inhibition of transcription following actinomycin D pre-treatment in both cases was able to negate the sPDZD2-induced increase in p53 activation. Functional siRNA knockdown of p53 minimised effects of exogenous sPDZD2 in both DU-145 and MCF-7 cells, and while silencing of p21 was able to reverse sPDZD2-induced mitotic arrest in DU-145 cells, no significant effect was observed in the MCF-7 lineage [92]. When taken together, these results indicate that autocrine secretion of sPDZD2 might represent a mechanism that, by direct transcriptional activation of p53, is able to initiate quiescence or senescence programmes that could contribute to induction or maintenance of dormancy in breast and prostate cancer. However, the impact of tumour cell residency in the bone on this putative mechanism, and the relevance of autocrine sPDZD2 secretion to cellular dormancy *in vivo*, remains to be elucidated.

SUMMARY

A significant body of evidence now exists which supports that microenvironmental regulation of tumour cell dormancy within the bone marrow is the product of mitotic arrest or suppression of oncogenic signalling resulting from signals derived from the niche in which DTCs reside (Figs. **2** and **3**). Many of the mechanisms implicated in maintaining

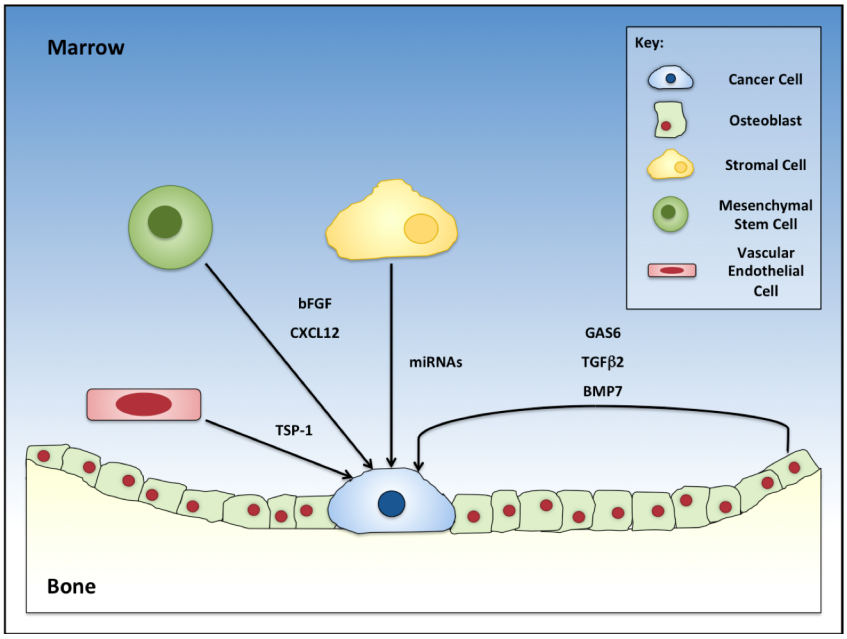

Fig. (2). Metastatic Niche Cells Contributing to Cellular Dormancy in Bone. A variety of cell-types comprise the metastatic niche in bone to which DTCs home and within which they reside during quiescence. Osteoblasts and mesenchymal stem cells secrete a multitude of paracrine and endocrine factors that, along with TSP-1 secreted by the endothelium of mature vasculature beds, interact with DTCs to modulate a number of pro-dormancy signal transduction pathways. Bone marrow stromal cells also contribute to this effect by paracrine shedding of miRNAs contained within exosomes. In addition, the formation of gap-junctions between DTCs and stromal cells, possibly induced during niche-selection, also facilitates import of quiescence-regulating miRNAs.

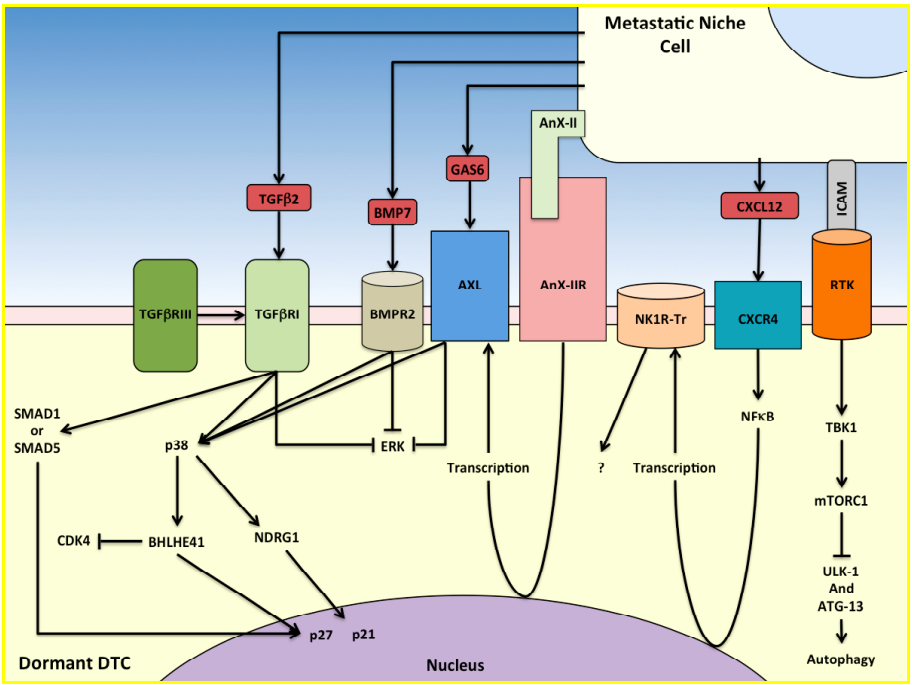

Fig. (3). Dormancy Inductive Signal Transduction Pathways. Cells that comprise the bone metastatic niche secrete a number of soluble factors, including BMP7, GAS6, CXCL12 and TGF-β2. In all cases, receptor ligation activates p38 signalling and inhibits ERK, inducing expression of BHLE41, NDRG1 and the cyclin-dependent kinase inhibitors p21 and p27, leading to cell-cycle arrest. This situation is further potentiated following up-regulation of AXL expression in response to ligation of annexin II receptors (AnX-IIR) on the cancer cell surface by annexin II (AnX-II) on stromal cells. Canonical signalling *via* SMAD1 or SMAD5 also induces p27 in response to TGF-β2. Binding interactions between DTCs and stromal cells induces TBK1 expression that, *via* negative regulation of mTORC1, results in the induction of autophagy that may facilitate long-term survival of dormant tumour cells.

cellular dormancy within the bone microenvironment are recapitulated quiescence programmes that normally direct adult stem cell biology. This provides a clear rationale for continued investigation of similar pathways in order to identify further putative targets for the development of therapeutic interventions aimed at prevention of bone metastasis.

The contemporary literature also contains compelling evidence for a number of dormancy-inductive and regulatory stimuli that might provide promising leads for future translational research. Amongst these, metabolic and nutritional stress or loss of adhesion-dependent signalling capability appear to be particularly important triggers of both stress-tolerance signals and autophagic pathways leading to a protracted state of cellular quiescence. However, many of these pathways were discovered in HNSCC models and are very poorly characterised in breast or prostate cancer. Definitive evidence for their role in acquisition and maintenance of the dormant phenotype in breast and prostate cancer will be required if the molecules and mechanisms involved are to be identified as novel therapeutic targets.

Understanding the molecular mechanisms involved in maintaining tumour dormancy is an important topic that is receiving widespread attention. As our insight into this area increases, the field may well become important for translational research, not only for eradicating dormant disseminated tumour cells from the bone, but also for maintaining certain tumours in a dormant state and thereby preventing their progression.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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