<table>
<thead>
<tr>
<th>Title</th>
<th>Experimental models of bone metastasis: Opportunities for the study of cancer dormancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Chong, Mark Seow Khoon</td>
</tr>
<tr>
<td>Date</td>
<td>2015</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10220/40559">http://hdl.handle.net/10220/40559</a></td>
</tr>
<tr>
<td>Rights</td>
<td>© 2016 Elsevier. This is the author created version of a work that has been peer reviewed and accepted for publication by Advanced Drug Delivery Reviews, Elsevier. It incorporates referee’s comments but changes resulting from the publishing process, such as copyediting, structural formatting, may not be reflected in this document. The published version is available at: [<a href="http://dx.doi.org/10.1016/j.addr.2014.12.007">http://dx.doi.org/10.1016/j.addr.2014.12.007</a>].</td>
</tr>
</tbody>
</table>
Experimental models of bone metastasis: Opportunities for the study of cancer dormancy

Chong Seow Khoon, Mark

School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore

Address:

Block N1.3, B3-13

62 Nanyang Drive

Singapore 637459

Email address:

markchong@ntu.edu.sg
Abstract

Skeletal metastasis is prevalent in many cancers, and has been the subject of intense research, yielding innovative models to study the multiple stages of metastasis. It is now evident that, in the early stages of metastatic spread, disseminated tumour cells in the bone undergo an extended period of growth arrest in response to the microenvironment, a phenomenon known as “dormancy”. Dormancy has been implicated with drug resistance, while enforced dormancy has also been seen as a radical method to control cancer, and engineering of dormant states has emerged as a novel clinical strategy. Understanding of the subject, however, is limited by the availability of models to describe early stages of metastatic spread. This mini-review provides a summary of experimental models currently being used in the study of bone metastasis, and the applications of these models in the study of dormancy. Current research in developing improved models is described, leading to a discussion of challenges involved in future developments.

Key Words

Metastasis, Experimental models, Cancer, Bone, Dormancy, Disseminated tumour cells
1 Motivation

Cancer dormancy refers to a protracted symptom-free period following the successful removal of a primary tumour until recurrence of the disease. Such clinical behaviour is commonly observed in cancers of the breast [1], skin [2] and prostate[3], with time to relapse ranging from years to decades. Recurrence is often manifested as metastatic lesions in distant tissue, particularly bone [4, 5]. Currently, the evidence suggests that metastasis-initiating cells are tumour cells that escaped from the primary site at an early stage of disease, disseminating to distant tissue, where they are initially clinically undetectable due to their small volumes. There, they remain undetectable due to microenvironmental cues that limit the proliferation of the “occult” metastatic tumour [6, 7], and the metastasis can only progress when the tumour is able to overcome these imposed metastatic blocks. Much research in the past decade has focussed on elucidating these mechanisms, in order to identify therapeutic targets that may limit the progression of the disease, as well as to understand the implications of dormancy on chemoresistance. Indeed, experiments on mouse models of breast cancer dormancy have confirmed quiescent cells to be refractory to conventional chemotherapy [8], and it was further suggested that chemotherapy was only able to eradicate tumour cells that had exited dormancy during treatment.

Despite the importance of understanding dormancy, advances in the field have thus far been severely limited by the scarcity of adequate cancer metastasis models, particularly those which are amenable to induction and monitoring of early post-metastatic events [9]. Indeed, the range of in vitro and in vivo experimental models available neither possess sufficient sophistication and finesse in recapitulating physiological processes during the early stages of metastasis, nor are they amenable to long-term serial, single-cell monitoring, required for the study of cancer cell dormancy. Thus, while classical models of cancer metastasis have proved integral in the dissecting the processes of metastatic invasion and dissemination [10, 11], a significant gap exists in recapitulating
the events between the steps of initial seeding and eventual outgrowth that encompass dormancy. Consequently, hypotheses on the governing mechanisms or even existence of metastatic dormancy have hitherto been largely built on mathematical models based on clinical data [12]. Serendipitously, technological advances have led to increasingly sophisticated models of metastatic disease being developed in recent years. In particular, much attention has been paid to replicating tissue-specific cues that influence cancer cell behaviour, in accordance with Piaget’s seed-soil theory, where metastatic “seeds” will only thrive in tissues with appropriate microenvironmental “soil” [13]. Bone, a common metastatic focus for many cancers [14], is one such tissue under intense scrutiny. Of note, the bone marrow is an established stem cell niche, with possible overlapping mechanisms governing dormancy of cancer and haemopoietic stem cells. [15]. It follows that novel models of bone metastasis may provide an avenue to study metastatic dormancy, and will be the subject of this review. This mini-review serves to provide a summary of in vitro and in vivo models commonly used in cancer metastasis studies, and to highlight the current research efforts being undertaken to tailor these models for the study of dormancy. Finally, the challenges that remain to be addressed are summarised, leading to future research directions. To facilitate the discussion, an overview of cancer dormancy and the bone marrow niche will first be described.

2 Dormancy and the role of the bone marrow

Solid tumours often undergo an extended period of very slow growth, in which time the patient presents little or no symptoms of the disease [5]. This is manifested in some patients who have undergone apparently successful treatment for cancer, only to develop overt metastasis, sometimes more than a decade later [16]. In other patients, minimal residual disease may persist in the form of Circulating Tumour Cells (CTC) in circulation even over twenty years after removal of the primary tumour [17]. Mathematical models describing the kinetics of the disease demonstrate that disease
progression is inconsistent with a continuous-growth model [18], instead showing that the natural history of the disease likely involves dormancy and metastatic reactivation. In the light of these findings, it has been suggested that cancer cells are disseminated to distant tissue early, where they remain dormant until activated, triggering metastatic relapse [19]. It has been proposed that two categories of dormancy exist: (1) Cellular dormancy, in which single cells enter into a non-proliferative state and (2) tumour-mass dormancy, where the growth of the tumour mass is limited by a state of matched turnover between proliferative and apoptotic cells. By this definition, cellular dormancy refers to a state in which the disseminated tumour cells (DTC) enter G0/G1 arrest, whereas tumour mass dormancy occurs where growth of micrometastases are limited by factors inducing cell death, including vascular insufficiencies and immune surveillance. The similarities with primary tumour dormancy are evident; the scope of this article will thus be limited to cellular dormancy as a critical step in the progression of metastatic disease.

As depicted in Figure 1, tumour cells are disseminated from the primary site, and are transported to distal tissue, where they get trapped singly. During these initial stages, endogenous pro-metastatic cues are balanced by exogenous microenvironmental cues limiting disease progression, which must be overcome in order for overt metastasis to develop. This microenvironment is largely the result of cells present in the bone marrow, including osteogenic cells and endothelial cells, as well as associated extra cellular matrix, which is thought to be initially unsuitable for cancer proliferation. Correspondingly, it has been reported that although occult lesions can be found in almost all healthy adults, only a fraction of these acquire malignancy [20]. For example, occult carcinomas have a prevalence rate of 99.9% in the thyroid, yet the incidence of thyroid cancer stands at only 0.1% [21]. It is thus evident that cancer cell response is largely dependent on interactions with the dissemination site and, in line with the cancer stem cell hypothesis, the concept of shared niche interactions with normal stem cells was developed [22]. For example, it has been established that osteoblasts and endothelial cells within the bone marrow perform a common role in the recruitment of Haematopoietic Stem Cell (HSC) and prostate cancer cells via Annexin II [23]. Corollary to this,
metastatic quiescence may be governed by similar cues dictating HSC dormancy in the stromal microenvironment. This was demonstrated by Shiozawa et al, where binding to Annexin II was succeeded by expression of growth arrest-specific 6 (GAS6) receptors on prostate cancer cell, inducing dormancy of the cancer cells [24] and effectively mirroring the HSC-niche interactions[25]. Similar observations were also made by the same team in their studies on acute lymphoblastic leukemias [26].

Within the bone marrow, two distinct stem cell niches may be identified: the endosteal niche and the perivascular niche [27]. The endosteal niche is composed primarily of mesenchymal stem cells (MSC) and osteoblasts, which perform central roles in regulating haematopoietic stem cells dormancy through stem / progenitor pathways [28]. It is of note that osteocytes derived from normal bones and those associated with metastatic disease present distinct transcriptional signatures, although it remains unclear if these changes were the cause or result of metastastic colonisation, nor if these changes impact stem cell behaviour [29]. Depletion of osteolineage cells is known to result in loss of HSC from the bone marrow and obtunds extramedullary haematopoiesis, further highlighting the links between the osteogenic and haematopoietic systems [30]. In a study by Greembaum et al, a similar effect could be achieved by CXCL-12 deletion in the bone marrow [31]; CXCL-12 is also implicated in HSC [32] and breast cancer cell quiescence [33]. Interestingly, when CXCL-12 deletion was performed selectively on specific cell populations in the bone marrow, it was found that modifying mineralising (mature) osteoblasts had no effect, whereas targeting the early mesenchymal progenitors resulted in constitutive mobilisation, leading to debate on the contribution of mature osteoblasts to HSC maintenance [31]. Other recent studies involving MSC demonstrate exchange of microRNAs between MSC and cancer cells via gap junctions [33] and exosome signalling [34] promote breast cancer cellular dormancy. BMP-7 is another secretory factor from bone stromal cells known to induce cancer dormancy [35]. Incubation of prostate cancer cells with BMP-7 was shown to activate p38, NDRG1, p21 and p21, while suppressing the ERK-MAPK pathway. P38 activation, in particular was found to drive NDRG1, a metastasis suppressor gene.
BMP-7 injected into a mouse model of skeletal metastasis effectively suppressed tumour growth; this effect was lost upon withdrawal of the BMP.

In contrast to the endosteal niche, the perivascular niche has conventionally been associated with activated HSC, although recent evidence suggests that almost all HSCs occupy a perivascular location, even within the endosteal region [36]. Similarly in cancers, metastatic tumour cells require a perivascular location in order to survive [37]. This niche is populated by endothelial cells (EC), MSC and other stromal cells characterised by a high CXCL-12 expression. Recently, EC have been shown to play a key role in HSC regulation, where EC-specific Scf deletion was found to deplete HSC from the bone marrow [38]. Crucially, it was shown that EC in stable microvasculature express thrombospondin-1, which was found to induce breast cancer cell quiescence in vitro and in zebrafish models [39]. In actively remodelling microvasculature, however, endothelial “tip” cells in emerging vascular sprouts upregulate TGFβ1 and periostin, accelerating cancer cell outgrowth.

In addition to the cellular players, the extracellular matrix (ECM) plays important roles in the progression of metastasis. For example, Tenascin C and periostin work in tandem to modulate Wnt signalling via the receptor Fzd7 [40, 41]. Fibronectin is another of several ECM genes upregulated in breast cancer metastasis [42] and has been shown to drive breast cancer cell proliferation through the activation of integrin-β1 [43]; this has been similarly demonstrated with Type I collagen [43]. Finally, ECM remodelling sees indirect effects on physical properties, such as matrix stiffness, with profound effects on cancer cells in the metastatic site, including epithelial-mesenchymal transitions [44], increased invasiveness [45] and proliferation[46]. It thus follows that the chemical and physical properties of the ECM are likely to have significant roles in governing cancer cell dormancy and metastatic activation.

3 Models of skeletal metastatic disease
As seen above, the microenvironment of the metastatic site is tightly implicated in cancer metastasis. The ability to recreate this environment is thus imperative for the development of disease models, in order to better represent the human condition. This task is complicated by the need to incorporate species-specific responses and heterocellular cross-talk. To further compound the problem, early events in metastasis occur at a single-cell level, asynchronously and randomly arriving at the metastatic site, creating technical challenges to monitor cellular responses. As such, classical models of metastasis are inadequate for studies on dormancy. The following section will discuss recent innovations of metastatic models, which may address some of these issues.

3.1 In vitro models

In vitro assays have been used for several decades and have been instrumental in studies on metastasis thus far. Many standardised assays are available to evaluate proliferation, apoptosis, motility and/or cellular invasion and share favourable characteristics of low cost, ease of handling, amenability to high resolution imaging and general accessibility for detailed downstream experimentation. In particular, Boyden chamber techniques are widely employed to study cancer cell migration [47]. Named after its inventor, the chamber was developed as a filter device that separates a culture well into upper and lower compartments. Cancer cells are typically added in the top compartment, allowed to migrate across the membrane in response to chemokines and migration rates taken as a measure of invasive potential. Modified chambers with coated ECM on the underside of the membrane have also been used to evaluate haptotaxis [48]. In the context of bone, breast cancer cell migration towards primary bone stromal cells seeded in the lower compartment has been demonstrated [49]. The set-up, however, is limited in providing kinetic data, as quantifications are typically performed at terminal end-points. In that aspect, scratch assays, where confluent monolayers of adherent tumour cells are disrupted by creating a linear scratch, offer real-time evaluation of cell motility, morphology and even protein localisation as the cells migrate to close the gap [50].
It is apparent, however, that these assays are typically used to study migration, and generally not amenable to studies on cell proliferation kinetics. As such, despite having been critical in furthering our understanding of cancer metastases, cancer cell migration assays have limited applications in the study of dormancy. In addition, another obvious limitation of such assays is the excessively simplified representation of the pre-metastatic bone microenvironment. As discussed above, the metastatic niche is a complex environment influenced by constituent cell types and ECM composition. In this light, the critical requirements of an ideal dormancy model includes (i) faithful anatomical reconstruction of the metastatic site (ii) recapitulation of early physiological events of cancer dissemination (iii) accessibility to data acquisition.

Recently, in efforts to better represent the myriad cell populations present in the metastatic niche, heterocellular “organotypic” culture models, in which a central role for EC in governing tumour cell dormancy could be demonstrated [39]. In their study, Ghajar et al created “microvascular niches” comprising endothelial cells and stromal cells derived from the bone marrow to represent the pre-metastatic niche (Figure 2). YFP-labelled cancer cells were added to the cultures, and it was observed that presence of the EC reduced proliferation five-fold. Single-cell monitoring revealed a perivascular location of the dormant cells on the vascular “stalks”, and that cancer cells found near vascular “tips” were more likely to proliferate. It follows that areas of active vascular remodelling are more susceptible to metastatic activation, and this was demonstrated in cultures with Notch-suppressed microvascular networks. With the increasing range of fluorescent labels available, such cultures may be further exploited to allow the concurrent imaging of multiple cell types, and integrated with image acquisition and analysis systems to yield greater amounts of information, as demonstrated by Chong et al [51].

Using tissue engineering techniques [52], in vitro models of vascularised bone were generated, on which prostate cancer cells were seeded. EC were labelled with GFP, and cancer cells were labelled with histone-tagged RFP, which facilitated the evaluation of vascular morphometry and proliferation respectively (Figure 32). Crucially, the data was obtained in real time, without need to terminate the experiments, enabling kinetic studies and
retrospective analysis of time lapsed images. Current work on improving this system includes incorporating fusion proteins, gene reporters and molecular beacons to allow the study of other signalling pathways in parallel [53, 54]. Such two-dimensional (2D) assays are rapidly generated without need for highly specialised equipment and may thus be most useful as drug screening platforms.

Recently, tissue engineering technology platforms have been translated into strategies to re-create a cell’s naturally occurring macro and microenvironment in order to study normal and pathological cell-cell and cell-niche interactions in vitro. In the case of cancer one might argue that the new field of tumor and tumour microenvironment engineering is an off-spring of biomaterial-based tissue engineering strategies [55]. Both the native and diseased tissues are three-dimensional entities, and ultimately the most representative culture model of any tissue must mirror this aspect. However, the effect of dimensionality on cells is complex to study and a means to do this has yet to be fully realized, making scaffold and hydrogel based in vitro model development to deconstruct and study individual niche components particularly attractive.

Like physiological tissue, the pathological behaviour of cancer cells in three-dimensional (3D) cultures is well-recognised to be vastly different from 2D cultures, and it is likely critical to reproduce the architectural complexity seen in the bone [56]. To this end, efforts have been made to develop bioreactors capable of supporting multiple-cell-layered osteogenic constructs to mimic 3D bone [57]. Breast cancer cells were then seeded, and observed to form a “single cell file” typical of metastatic cancers in vivo. Cancer cell proliferation rate found to be reduced in 3D, while osteoblasts in the coculture altered phenotype in response to the metastatic invasion, adopting a more cuboidal morphology [58]. In another model of metastatic bone, Marlow et al grew MSC together with EC in a collagenous matrix to generate a 3D coculture model, observing that monocultures of MSC supported proliferation of subsequently seeded breast cancer cells, whereas MSC-EC co-cultures were inhibitory [59]. Cell cycle-arrested cells could be rescued by
removing them from the inhibitory niche, suggesting the validity of system as a true model of cancer dormancy. It also follows that conventional tissue engineering approaches can be used to extend these constructs into millimetre scales [60], where co-cultures of endothelial and mesenchymal stem cells are loaded via a fibrin gel carrier into a synthetic polymeric scaffold structure. In conjunction, bioreactors may be used to overcome mass transport issues in order to maintain the viability of such engineered constructs [61].

It should be noted here that the cell/material interface in engineered tissue models is a complex, dynamic microenvironment in which reciprocal interactions between the cell and the material cooperatively dictate mutual fates: the cell by remodeling its surroundings, and the material through its inherent properties. Extrinsic regulation of tissue elements - including cell-cell contact mediators, secreted signaling factors, extracellular matrix (ECM), substrate stiffness and topography, nutritional parameters such as oxygen and transport of nutrients and metabolized waste products, pH, temperature, fluid flow, mechanical stress – may be evaluated and/or manipulated in three-dimensions to generate a modular toolbox. As an example, polymeric scaffolds may be used to provide a guiding template for seeded cells to grow on, giving rise to the stroma component within a provided porous architecture [62]. In parallel, cancer cells may be embedded in hydrogels, with the fundamental aim of mimicking crucial properties of the natural extracellular matrix (ECM), including the representation of integrin cell-binding motifs for integrin engagement and protease cleavage sequences to allow degradation by cell-secreted proteases (Figure 42). In addition, the advantage of well-bioengineered hydrogels is that they can provide the needed modularity to change the mechano-biological characteristics depending on the biological hypotheses to be tested [63, 64]. Using this strategy, Sieh et al were able to show that engineered bone constructs were able to elicit osteomimetic behaviour and, more interestingly, modulated androgen-responsive genes in prostate cancer cells [65].
Going in the opposite direction, the approach can also be miniaturised using microfluidics devices. Microfluidic technology typically involves channels with dimensions in the range of micrometers to generate well-defined laminar flows, with precise control over geometric and temporal profiles of soluble factors. Using a two-layer microfluidic system, Hsiao et al created 3D multicellular spheroids comprising prostate cancer cells, osteoblasts and EC [66]. The method was shown to generate uniform spheroids which were amenable to high resolution imaging.

Exploiting the flow system further, Sung et al described the use of microfluidic flows to create patterned fibroblast–breast cancer cell interfaces, in order to study heterocellular interactions [67]. Using this platform, the authors were able to demonstrate a contact-dependent transition of ductal carcinoma in situ (DCIS) cancer cells into invasive phenotypes. Functional changes in the extracellular matrix was also possible with the use of second harmonics to evaluate collagen deposition. Microfluidic platforms, as demonstrated by the Kamm group, can also be used to mimic the haematogenous route taken by metastatic cancer cells [68] (See Figure 5). By assembling endothelial cells on the walls of the microchannels and/or through a collagenous gel region, an interconnected microvasculature could be formed, through which tumour cells could be flowed through [69]. Extravasation rates could be captured, and, more crucially, high resolution, real time imaging could be performed to characterise single cell dynamics in the complex microenvironment. This biomimetic device can used to study factors influencing dormancy, or to validate effects of anti-metastatic drugs [70]. Similar research and development have also been undertaken the Beebe group, where they demonstrated formation of patterned, interconnected endothelial networks through the hydrogel region [71]. Other variations that can be made include incorporation of cytokines to establish defined profiles in the device to study cell migration [72], angiogenic factors to stimulate vessel formation [73] and even control over oxygen availability to mimic hypoxic conditions [74]. These studies serve as examples to show the use of microdevices to generate well-defined microenvironments, which will allow the dissection of molecular mechanisms and contributing factors surrounding dormancy.
3.2 In vivo models
While the in vitro models above deliver unsurpassed control over experimental conditions in order to investigate specific facets of metastasis, they are inevitably unable to fully recapitulate the complex interplay of various tissue systems in metastasis. In particular, interactions between cancer cells and stroma are dynamic and involve systemic host responses. In this aspect, animal models are critical for furthering our understanding of the disease. Of all animal species, arguably the most widely studied is the laboratory mouse. Laboratory mice bear many physiological and genetic similarities to humans, and are thus used as model systems in many diseases. Additionally, tools are readily available for the genetic manipulation of mice, to allow establishment of the molecular events of human cancer pathogenesis in the mouse or, through the use of reporter genes, to track the progression of cellular and molecular interactions within the tissue microenvironment. [73]

The ultimate model will be able to establish the multiple stages of metastasis in vivo in a single mouse, and several strains of genetically engineered mice (GEM) have been generated explicitly with this intention. These GEM are typically engineered for transgenic expression of oncogenes or knockout of tumour suppressor genes, and range from spontaneous tumour progression models to inducible or conditional models. Using such techniques, latency has been observed in the RET.AAD model of melanoma, albeit induced by tumour mass dormancy rather than cellular dormancy [75]. The single GEM capable of replicating all clinical features of human malignancy, however, remains largely elusive [76], and spontaneous orthotrophic metastases are particularly rare in GEM models [77]. To address this issue, one strategy involves harvesting spontaneous tumours with high metastatic potential, followed by syngeneic transplant or orthotopic injections of cancer cells into a new host. Serial transplantations are also commonly performed to select for tissue-trophic sublines. As an example, the highly metastatic 4T1 carcinoma cell line was derived from a spontaneous mouse mammary tumour. Through serial transplantation and selection for the most metastatic clones, the 4T1.2 line was generated; this can then be implanted into mammary pads of news hosts to generate
tumours with high metastatic frequencies to bone, lung and lymph node [78]. Using such models, Li et al demonstrated distant retardation of metastatic growth by the primary tumour and that metastatic growth could be reinstated after resection of primary tumour [79]. Similar findings made by other groups have been used to build a case for the role of the primary tumour in contributing to DTC dormancy, although questions remain over the relevance of these observations to human settings [80]. Additionally, it should be noted that orthotopic models generally have limited applications in the study of dormancy, as the timing of metastatic onset remains random, rendering this model impractical for kinetic studies. To address this, cancer cells are often delivered into circulation or directly into osseous tissue. Intracardiac injection of a subline of B16 murine melanoma cells, for example, has been shown to reproducibly generate metastatic lesions in the spine and long bones [81]. This, however, is an exception rather than the norm, as intracardiac injections rarely result in bone metastatic formation. Consequently, intra-tibial injections are more commonly performed as models of “metastatic” lesions in the skeleton, but do not recapitulate early events in metastasis [73]. It follows that existing GEM models would have limited applications in aiding our studies on dormancy, and are particularly handicapped in recapitulating human features of the disease.

The advent of immunocompromised mice opened new avenues to investigate human cancer-specific responses. Designed for xenogeneic implantations, human cancer lines can now be injected directly into a murine host, in order to better reproduce human pathophysiologies. In such experiments, immunocompromised strains, such as NOD / SCID, are injected with human cancer cells, either through an intracardiac route, or into an orthotopic location [82]. Cancer cell numbers in distant tissue may then be quantified by harvesting the tissue in question followed by real time polymerase chain reaction (qPCR) [83]. The cancer cell lines used in such studies may also be genetically modified in knock-in / knock-out studies to improve our understanding of molecular processes in metastasis. In their study, Chu et al reported roles for RANK and c-Met in the metastatic activation of
disseminated prostate cancer cells in the bone [84]. Recent innovations in imaging of bone tissue have also opened up new possibilities to track and monitor cancer cell proliferation at higher resolutions in real time [85]. While primarily used to evaluate metastatic invasion, these models may be coupled with “chase” experiments to study dormancy [86].

Perhaps of greater significance is the opportunity to create even more “humanised” mice as models of the disease. As reviewed recently by Holzapfel et al, humanised xenograft models have important implications in reproducing species-specific aspects of tumour interactions [87]. This is highlighted by experiments showing preferential tropism of cancer cells for human bone subcutaneously implanted in SCID mice [88]. It was thus proposed that humanised models comprising cancer cells and pre-metastatic tissue of human origin would have greater clinical relevance. Hence, breast cancer cells could be found to metastasise towards implanted tissue engineered bone in a mouse model, at rates dependent on the osteogenic state of the tissue construct [89]; similar results could be observed using prostate cancer lines [90]. Strikingly, as seen in Figure 64, implanted engineered bone constructs were found to acquire a marrow-like tissue containing haematopoietic progenitors, reminiscent of the endosteal niche [91]. These “organoids” remain viable and are vascularised in vivo, leading to recapitulation of actual metastatic events in cancer, including cancer cell engraftment and osteolytic degradation. Such systems may be altered to study cancer cell sensitivity to osteogenic states [89] or decellularised matrices may be generated [92] and implanted to evaluate ECM-specific influences. Tumour cell migration and proliferation, and skeletal remodelling may then be interrogated longitudinally, aided by their extra-skeletal location, and close proximity to skin [93]. Other advantages of this system include amenability of the construct to genetic manipulation prior to implantation. The use of tissue engineered bone in a metastasis model will thus allow the generation of a range pre-metastatic conditions, followed by longitudinal and real-time assessment of critical cancer parameters.
In spite of these advances, however, it can be observed from the above that the following conundrum exists. In vivo models provide a complex environment comprising unique interactions that cannot be replicated in vitro. Yet, the same models face limitations dissecting the complex and different stages of metastases step by step (e.g., single-cell responses). A recent innovation that seeks to address this problem is the “bone marrow on a chip” [94]. Using techniques similar to those outlined above, Torisawa et al. describe the retrieval of a subcutaneously implanted tissue engineered bone construct, followed subsequently by transfer to a microfluidic device designed to sustain the viability of the biopsied bone marrow. Haemopoietic stem cells could be grown in these devices, suggesting preservation of the haemopoietic niche. Such organs-on-chips thus marry the merits of the complex environment yielded by in vivo tissue, while affording the control and amenability to high resolution imaging of in vitro systems.

In parallel, improvements in intravital imaging has led to increasingly sophisticated models amenable to serial live-imaging, allowing longitudinal studies. In their study, Wolf et al. employed multiphoton microscopy to track labelled tumour cells in a dermal chamber [95]. Using second-harmonics, they were able to simultaneously visualise connective tissue, showing amoeboid migration of cancer cells through tissue space. Other developments, such as the “femur window” may also be useful in this aspect. In their paper, Hane-Algenstaedt et al. described the development and use of a bone chamber implant for in vivo microscopic examination of the bone tissue and microcirculation for up to 90 days [96]. Using this system, leukocyte-endothelial interactions and cancer cell engraftment could be observed. Similar models have already been exploited to study cancer metastasis to the brain [37].

**4 Challenges**

Significant advances have been made in generating in vitro and/or in vivo cancer models of skeletal metastasis. Even with the wealth of in vitro and in vivo models currently available, however, many
challenges remain for them to be applicable to studying the important feature of cancer, namely
dormancy. Firstly, cellular dormancy remains poorly understood, and “dormant cells” can only be
retrospectively defined by their inability to proliferate. Depending on the assay system, purported
dormant cells may be mislabelled in situations of matched turnover, senescence, permanent cell-cycle
arrest or delayed growth kinetics. Improved definitions of dormant cells are needed, as are methods
to identify the bona fide dormant cell, including definitive markers or profiles of receptors, in order to
facilitate the monitoring of dormant cells within the microenvironment. Standardised functional assays
may be useful in this aspect as well. It should be noted at this point that this is perhaps a Catch-22
situation, given that dormant cells are inherently rare and difficult to isolate, impeding efforts to
characterise them fully [97]. In a recent report by Chery et al, expression profiling of DTC harvested from
the bone marrow of prostate cancer patients revealed distinct expressed gene sets in patients with no
evidence of (or minimal residual) disease versus those with advanced disease [98]. Strikingly, members
of the p38 pathway were identified, which may potentially be used as biomarkers of metastatic
activation.

A related problem is that of data acquisition. Metastasis, as described above, is a rare and
asynchronous event initiated by single cells, while dormancy is defined by extended periods of little
or no growth. Taken together, this suggests the need to repeatedly scrutinise large volumes of tissue
in order to register early events, followed by long term monitoring to track the progression of
dormancy, rendering most existing models unwieldy and impracticable. There is thus a need to
develop high-throughput, more sensitive methods capable of detecting dormant cells. Following
identification and isolation, improved techniques will also be needed to carry out further analyses at a
single cell level, and technologies enabling real-time, multi-parameter cell analyses are required.
Finally, it should be noted that the models discussed here have hitherto largely been qualified only in
the general context of skeletal metastasis. Cross-validation for use as dormancy models with clinical
data are a conditio sine qua non.
5 Conclusions

Dormancy is an emerging area of cancer research severely limited by the lack of available models. Recently developed models of skeletal metastasis may be helpful in addressing this gap. These range from in vitro systems complemented with real-time, high resolution imaging system to in vivo models capable of faithful recapitulation of clinical pathophysiologies. The applications of these models in the study of cancer cell dormancy will be integral in advancing our understanding of the phenomenon, and raises interesting possibilities for development and evaluations of new cancer therapies.

6 Acknowledgments

The author would like to gratefully acknowledge support by the National Medical Research Council (NMRC) Cooperative Basic Research Grant (CBRG) BNIG12nov009 and the Ministry of Education Tier 1 Academic Research Fund 2013-T1-001-046.

7 References


8 List of Figures

Figure 1: Schematic overview of the metastasis process. Tumour cells disseminate singly and asynchronously from the primary site, often to a distal bone, where they occupy the haemopoietic niche in bone marrow. There, interactions between the disseminated tumour cell (DTC) and the microenvironment (including osteogenic, vascular cells and associated extra cellular matrix) govern DTC decision to enter dormancy or metastatic activation. Several experimental models have been developed to allow scientists to study cancer cell escape and intravasation, as well as metastatic tumour growths. The conventional models, however, have limited use for studies on cellular dormancy. This figure was produced using Servier Medical Art (www.servier.com).

Figure 2: Co-cultures of YFPlabelled breast cancer carcinoma cells (T4-2; white), endothelial cells (CD31; red) and bone marrow stromal cells (HS-5). Ki-67 was used to identify proliferating cells. Stable endothelia were found to inhibit, whereas sprouting vasculature were found to promote T4-2 cell proliferation. Scatter plot shows dwell-time fraction (which gives an indication of aggregate time spent in a location) versus division time (time taken before undergoing division), and demonstrates that cell decision to divide is dependent on proximity to neovasculature.

Figure 3: Organotypic cultures comprising endothelial cells (EC; green) and mesenchymal stem cells (MSC) to represent vascularised bone. Prostate cancer cells (PC3; red nuclei) were subsequently added to the resultant system. High resolution images taken three days post seeding show the emergence of tight clusters of PC3 cells sparsely distributed throughout culture system (indicated by yellow arrowheads). Additionally, quiescent cells were sporadically found to be singly adhered. Reprinted with permission from Chong et al, Mol Pharm, 11 (2014) 2126-2133. Copyright 2014 American Chemical Society.

Figure 4: Tissue engineered 3D co-culture model and the effect of co-culturing LNCaP cells with TEB. Prostate cancer LNCaP cells were encapsulated in hydrogels and cultured in the absence or presence of 3D engineered bone. More colonies with smaller size were formed in the co-cultures as compared to monocultures. Scale bar: 100 μm. Reprinted with permission from Sieh et al, Bone, 63 (2014) 121-131 with permission from Elsevier

Figure 5: Microfluidic 3D in vitro model of breast cancer metastasis to bone. (A) Osteo-cells were differentiated from MSC, harvested and seeded within the gel region of a microfluidic device to generate mineralised matrix. EC were then added to the media channel to generate a monolayer representing the capillary wall. Cancer cells were then flowed through the media channel, and (B) allowed to spontaneously extravasate into the gel region (C) Osteo-cell conditioned environment was found to favour extravasation over a collagen gel control. Reprinted with permission from Bersini et al, Biomaterials, 35 (2014) 2454-2461, with permission from Elsevier

Figure 6: Humanised moused model of bone metastasis (A) Tubular scaffolds produced by melt-electrospinning were seeded with human mesenchymal progenitor cells (B) SEM imaging demonstrated that hMPCs had formed dense sheets covering the entire scaffold (C) Following implantation into NOD/SCID mice, the tissue engineered ossicle (asterisk) had a dark red colour.
Extensive neovascularisation (arrows) was evident around the construct. (D) Bone marrow spaces included cellular components of proliferative haematopoietic marrow. Well-expanded sinusoids filled with red blood cells (asterisk) were scattered within the marrow cavity and were surrounded by haematopoietic cells of different lineages. Megakaryocytes were identified as large cells with lobulated nuclei which were attached to the perisinusoidal wall (arrows). (E) Human prostate cancer cells were injected into the left ventricle, and weekly BLI showed the first localized signal at the left flank one week after cancer cell inoculation. After 4 weeks multiple metastatic foci resulted in a summarizing signal. Reprinted with permission from Holzapfel et al., Biomaterials, 35 (2014) 4108-4115, with permission from Elsevier.