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Thorny ground, rocky soil: tissue-specific mechanisms of tumor dormancy and relapse

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Abstract

Disseminated tumor cells (DTCs) spread systemically yet distinct patterns of metastasis indicate a range of tissue susceptibility to metastatic colonization. Distinctions between permissive and suppressive tissues are still being elucidated at cellular and molecular levels. Although there is a growing appreciation for the role of the microenvironment in regulating metastatic success, we have a limited understanding of how diverse tissues regulate DTC dormancy, the state of reversible quiescence and subsequent awakening thought to contribute to delayed relapse. Several themes of microenvironmental regulation of dormancy are beginning to emerge, including vascular association, co-option of pre-existing niches, metabolic adaptation, and immune evasion, with tissue-specific nuances. Conversely, DTC awakening is often associated with injury or inflammation-induced activation of the stroma, promoting a proliferative environment with DTCs following suit. We review what is known about tissue-specific regulation of tumor dormancy on a tissue-by-tissue basis, profiling met major metastatic organs including the bone, lung, brain, liver, and lymph node. An aerial view of the barriers to metastatic growth may reveal common targets and dependencies to inform the therapeutic prevention of relapse.

Keywords

disseminated tumor cell dormancy; microenvironment; dormant niche; metastasis; quiescence

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Conflict of Interest statement

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Introduction

In his writings on cancer, the Greek physician Celsus (25 BC-50 AD) remarked on patients who, "…attain to a ripe old age in spite of it. No one, however, except by time and experiment, can have the skill to distinguish a cacoethes which admits of being treated from a carcinoma which does not" [1]. Even today, with a plethora of advanced technology and medical knowledge, this statement rings true; a cancer diagnosis may no longer be a death sentence, but distinguishing between a cancer that requires vigilant management from one that will no longer pose a problem remains a distinct challenge.

In the millennia following Celsus, massive progress has been made in the areas of cancer prevention, prognosis, and treatment; yet metastasis continues to be a significant setback in patient health, with few treatments aimed at its specific nuances and presentation, A female patient who presents with a tumor in the breast has a 99% 5-year relative survival rate, which drops to 27% if she has distant metastases at that time (SEER database, 2010–2015, National Cancer Institute). The outlook worsens if the tumor is of the treatment-refractory triple-negative subtype, with a 5-year survival rate of 91% for localized disease, which drops to 11% with distant metastases. Analyses of matched primary and metastatic samples demonstrate that metastases share genomic alterations and may switch subtype when compared to the primary tumor [2–4]. The evolution of metastases correlates with time to detection, therapeutic selection, and tissue-specific alterations [5–10]. It would not be going too far to state that metastatic disease should be assessed independently from the tumor of origin.

Tumor dormancy is a piece of the metastatic puzzle yet to be fully understood. Although it has never been observed directly in humans that metastases arise from dormant DTCs, dissemination occurs early in tumor progression [11–14], and experimental models have demonstrated that single, quiescent DTCs initiate metastasis [15–19]. In models of breast cancer, DTCs can survive for long periods of time (at least 240 days for D2.0R mammary carcinoma cells [16]) as quiescent cells in organs like the lung and bone marrow; after isolation from the tissue, formerly quiescent DTCs including M4A4 and NM2C5 (clonally derived from the MDA-MB-435 breast cancer pleural effusion cell line) have been shown to resume proliferation and expand in culture, demonstrating that at least a portion of DTCs retain the capacity for proliferation [20–22]. These key data demonstrate convincingly that tumor cells capable of growth in idealized conditions (cell culture, the orthotopic site) are constrained at some secondary locations. The nature of these restrictive mechanisms may be cell-intrinsic, as acquisition of specific mutations may be required for colonizing a new landscape [13, 23]; extrinsic, via suppressive factors or lack of proliferative signals provided by the niche that limit DTC colonization; or a combination of the two, in which niche-derived signals differentially effect early versus late DTCs, reflecting genetic changes acquired by DTCs over time [24].

From a clinical perspective, the dormancy phase between treatment and metastatic emergence is a lull in disease progression that holds a measure of relief and a promise of reprieve. It may last a year, decades, or a lifetime. For the significant proportion of patients who experience delayed relapse, this is a golden window of opportunity to

therapeutically intervene against future metastases. But first, we must capitalize on a wealth of historical observation and modern experimental data describing the biological and anatomical restrictions on tumor growth.

Rupert Willis formally introduced the concept of "tumor dormancy" in 1934 to account for seemingly inert tumor cells observed in autopsied tissues [25]. He was not the first to document these ectopic cells that seemed to contradict cancer's defining quality of voracious and unbridled growth. The growing consensus among clinicians was that, upon careful examination only possible during autopsies, tumor cells could be found throughout most tissues and in far greater numbers than metastases [26]. Ernst Fuchs' idea of tissue predisposition when describing uveal melanoma metastasis [27] and Stephen Paget's seed and soil hypothesis [26] helped frame in broad terms that certain tissues are more amenable to metastasis than others depending on cancer type; yet, the observation that many tissues contain isolated tumor cells, hereafter referred to as disseminated tumor cells (DTCs), argued that colonization, rather than dissemination, is the limiting factor [26]. Twenty years after Willis, Geoffrey Hadfield noted in 1954 that, "The anatomical site of these minute quiescent growths must be significant, for in at least 50% of cases of malignant disease recurring after latent periods…was found in the hemopoietic bone marrow" [28]. This is one of many pieces of evidence that metastases display a tissue preference [29]. But what makes a tissue fertile or infertile soil? Can an infertile soil become more amenable to growth, either through changes to the soil or within the seed itself? And, in a therapeutic context, can a permissive soil be made more hostile to DTCs? These questions have significant impact on our understanding of tumor biology and how we approach this potent stage of disease.

Tumor dormancy: A parable of the microenvironment

When considering the suitability of tissue for metastatic colonization, an agricultural analogy beyond Paget's "seed and soil" may be apt:

> "'A sower went out to sow his seed; and as he sowed, some fell on the path and was trampled on, and the birds of the air ate it up. Some f n on the rock; and as it grew up, it withered for lack of moisture. Some fell among thorns, and the thorns grew with it and choked it. Some fell into good soil, and when it grew, it produced a hundredfold.'" (Luke 8:5–8, NRSV)

Predators, insufficient nutrients, impediments to growth: these factors antagonize rampant DTC proliferation. DTCs are killed through direct elimination (immune editing) or fail to grow due to the absence of a hospitable niche and/or presence of anti-growth "morphostats" [30]. This review focuses on external *tissue-specific* factors and properties that impede metastasis through induction of cellular dormancy, defined as exiting the cell cycle into G_0 phase (quiescence) or extremely slow cell division, and the conditions that break down these barriers to allow metastatic growth. Although each tissue is distinct, several themes emerge from an aerial view that provide insight into major factors that regulate DTC dormancy and its loss, providing clues for future studies.

1. Bone

1.1 Metastasis to the bone

> "The evidence seems to me irresistible that in cancer of the breast the bones suffer in a special way…" –Stephen Paget, 1889 [26]

The bones are the third most common site of all solid tumor metastasis after liver and lung [31], reflected in the high rate of incidence of bone metastasis at diagnosis [32] and 10-year follow up [33]. A meta-analysis of SEER bone metastasis data found that 88.4% of metastatic prostate cancer patients have detectable bone metastases at diagnosis, followed by breast (53.71%), renal (38.65%), and lung adenocarcinoma (36.86%) [32]. Bone metastasis results in the painful destruction of hard bone and nerves, soft tissue inflammation, risk of fracture, and debilitation of the hematopoietic stem cell pool.

Tumor cell dissemination to the bone occurs frequently, as marrow aspirates reveal that 15.0–30.6% of breast cancer patients [34–37], and 57% of prostate cancer patients [38] who show no other evidence of disease harbor bone DTCs or micrometastases, and these correlate with worsened outcomes. The prevalence of DTCs in the bone has been attributed to wo factors: 1) the low blood pressure system of the sinusoidal beds, which aids in DTC extravasation [39]; and 2) DTC homing to the bone marrow via chemoattractant gradients including CXCL12 (SDF-1) via CXCR4 [40, 41]. The presence of DTCs in the bone predicts late relapse at that site and elsewhere [34, 35, 37, 42–44]; however, the discrepancy between high incidence of bone DTCs and relatively few detectable metastases in clinical observation and pre-clinical models [19] suggests growth-restrictive mechanisms in this niche.

1.2 The bone microenvironment

The bone is composed of hard, cancellous bone matrix surrounding an inner medullary cavity filled with vascularized fatty marrow. The inner surface of the hard bone, the endosteum, is a dynamic surface for cell interactions. It undergoes cycles of bone absorption and remodeling mediated by osteoclasts and osteoblasts, respectively, at punctuated sites across an otherwise homeostatic landscape [45, 46]. Arteries pass through the trabecular bone to the central cavity, emerging from the endosteum as arterioles. The endosteum therefore interfaces closely with arterioles, and associated cells including nestin promoter $(Nes)^+$ neural/glia antigen 2 $(NG2)^+$ αSMA^+ mesenchymal stem cells (MSCs) [47], sympathetic neurons and associated non-myelinating Schwann cells [48], adipocytes, and bone-specific immune cells [49–51]. Arterioles extend into the central cavity where they transition into venous sinusoids which serve as the route of egress for mobilized bone marrow cells (Fig. 1). Sinusoidal vessels form a distinct vascular niche that includes immune cells and several lineages of mesenchymal-derived cells bearing overlapping markers: CXCL12-abundant reticular (CAR) cells [52, 53], leptin receptor $(LepR)^+$ cells, Nes^+ cells, and $NG2^+$ cells, collectively referred to here as MSCs (reviewed extensively in [54]) (Fig. 1).

Another critical resident of the bone marrow is the hematopoietic stem cell (HSC), long-lived and reversibly quiescent stem cells that populate the blood lineages. The HSC niche is powerfully quiescent, and HSCs can be found in endosteal [55–59], arteriolar [60],

and sinusoidal [59, 61, 62] compartments under the influence of osteoblastic cells, MSCs, neurons, and endothelia. The HSC capacity for reversible quiescence while maintaining stemness is remarkably similar to properties of dormant DTCs, and therefore many studies have investigated parallel mechanisms of regulation (reviewed in [63]).

1.3 Mechanisms of DTC dormancy in the bone

Animal models show that the endosteal niche [19, 40] and the perivascular niche [61, 64–66] harbor myeloma, prostate, and breast cancer DTCs among others, and identifying the specific interactions that enable and regulate tumor dormancy is the next critical step. There is substantial overlap between the endosteal niche and endothelial niche considering that the endosteum interfaces with arterioles (reportedly, 90% of osteoblasts are within 20 μm of a blood vessel [67]), but unique features of each niche drive distinct quiescence programs.

The endosteal niche—The endosteum is a primary niche for HSCs and can be co-opted by DTCs. Osteoblasts secrete pro-quiescence factors like osteopontin [68, 69] and growth arrest specific-6 (GAS6), which regulates cell division through binding specific TAM (Tyro3/Sk, Axl, Mer)-family receptor kinases: Tyro3 induces cell division while Axl promotes quiescence via ERK1/2 signaling, survival, and chemoresistance [70, 71]. Notably, slow-cycling prostate cancer cells [71] and quiescent multiple myeloma cells [19] in the bone express high levels of Axl. Prostate cancer DTCs that bind to osteoblasts [72] or osteoclasts [70] upregulate Axl, which is then stabilized by the hypoxic environment of the endosteal niche [73]. Despite its anti-proliferative effects, Axl cannot maintain long-term dormancy on its own, indicating that it collaborates with other quiescence drivers [74].

Osteoblasts also secrete transforming growth factor beta (TGF-β) family members, which suppress tumor progression at early stages through activation of cyclin-dependent kinase (CDK) inhibitors [75–77] (reviewed in the tum or dormancy context in [78]). TGF-β1 TGF-β1 is a key component of the vicious cycle of bone metastasis [79]; conversely, TGF-β2 limits DTC growth. Osteoblast-derived TGF-β2 induced quiescence in prostate cancer DTCs through TGF-β receptor RI (TβRI) and TβRIII [80], similar to its effect on head and neck squamous cell carcinoma DTCs through p38α/β, p27, and DEC2 upregulation and CDK4 downregulation [18]. Prostate cancer DTCs themselves express elevated levels of TGF-β2 mRNA [81], which can be induced by Axl-GAS6 binding [72]. Endosteal $NG2^{+}$/$Nestin^{+}$ MSCs produce TGF-β2 and BMP7, which promote breast and head and neck squamous cell carcinoma (HNSCC) DTC dormancy in bone [82]. Estrogen-receptor positive breast cancer patients with high levels of TGF-β2 and BMP7 in the bone marrow experience decreased risk of systemic recurrence and prolonged metastasis-free survival [82]. These studies collectively support the quiescence-promoting role of bone marrow TGF-β2 signaling and suggest therapeutic avenues to restrict metastatic growth.

In addition to TGF-β2, MSC-derived BMP7 promotes a quiescent epithelial phenotype for prostate cancer DTCs [83] through a Smad-independent pathway involving p38, p21, p27, and NDRG1 [84]. Intriguingly, MSC BMP7 can be induced by SPARC secreted by indolent prostate cancer DTCs, revealing that DTCs actively elicit support from their microenvironment [85]. MSCs also secrete CXCL12, which promotes quiescence of chronic

myeloid leukemia stem cells [86], on top of its well-studied roles in bone marrow trafficking and HSC maintenance [87]. MSC-derived exosomes containing microRNAs like miRNA-23b [88] and miRNA-222/223 [89] induced quiescence and chemoresistance in breast cancer cells in culture and *in vivo.* The breadth of mechanisms through which MSCs interact with DTCs underscores their diverse functions as master regulators and maintainers of homeostasis within the bone microenvironment (Fig. 1).

Bidirectional interactions between DTCs and their niche have been identified in myeloma [19], breast [90], and prostate cancer [72, 85]. Breast cancer DTCs bias osteoblasts through direct contact to decrease secretions of IL-6, MMP3, and type I collagen [90], molecules shown to elicit proliferation [91, 92]. Direct co-culture of tumor-experienced osteoblasts and breast cancer cells results in increased p21 in the cancer cells, indicating that DTC-associated osteoblasts induce DTC cell cycle arrest through direct signaling and altered ECM deposition [90].

Acellular factors like oxygen tension also affect DTC behavior. Despite high vascularization throughout the bone marrow, hypoxic regions along the endosteum support HSC stemness by minimizing DNA damage from free radicals [93–95]. However, hypoxia may also promote escape from dormancy by inducing angiogenesis [66] and reposing leukemia inhibitory factor receptor (LIFR) in a hypoxia-induced factor (HIF)-independent manner [93]. LIF ligand is an IL-6-family cytokine that promotes proliferation in primary tumors [96] but inhibits DTC proliferation in the bone [93]. LIFR knockdown in different breast cancer cell lines correlated with decreased expression of quiescence and sternness genes including thrombospondin-1 (TSP-1), TGF-β2, and p53, as well as increased expression of proliferative genes c-Myc and pSrc, suggesting that LIFR is an environmentally-tuned gatekeeper for dormancy [93]. The seemingly contradictory role of hypoxia in the stem cell versus DTC niches reflects a nuanced balance between protection from free radical damage versus hypoxic stress.

The perivascular niche—In bone marrow, the perivascular niche refers to the microenvironment in and around arterioles concentrated at the endosteum and the sinusoids located in the central bone marrow cavity [97]. Arterioles are tight, VE-cadherin^{hi} vessels supported by NG2^{+} MSCs, sympathetic nerves, and associated non-myelinating Schwann cells, whereas sinusoids are fenestrated, leaky vessels supported by CAR^{+} LepR^{+} MSCs. There is evidence that both niches support HSCs: quiescent HSCs depart from arterioles when activated by radiation injury [98] and in a mouse model of overactive HSCs [60]; in contrast, up to 85% of HSCs may be perisinusoidal [59, 61]. Differences in vessel permeability regulate HSC differentiation via ROS, with ROS^{low} arterioles maintaining HSC stemness and ROS^{high} sinusoids promoting HSC activation [99]. In the tumor context, spontaneously dormant DTCs preferentially reside in the perisinusoidal niche in the calvarium, whereas micrometastases are only detected in non-sinusoidal, non-perivascular regions [65]. Although these studies do not resolve the discrepancy between the arteriolar and sinusoidal niches, they provide strong evidence that the perivascular niche is critical for maintaining DTC dormancy.

Endothelia comprising blood vessels are the first residents of a tissue to interact with extravasating DTCs. Mouse models show that quiescent MDA-MB-231 breast cancer DTCs preferentially reside on the basal side of bone marrow vasculature [66] where they are exposed to quiescence factors including TSP-1, TGF-β2, and BMP7 [82, 84, 100]. The perivascular niche chemoprotects DTCs through integrin signaling, in a manner distinct from quiescence regulation [101]. Doxorubicin treatment selected for perivascular DTC survival, and knocking down integrins β1 or αvβ3 in tumor cells or their ligands (VCAM-1 or von Willebrand Factor, respectively) in an organotypic co-culture model of the bone marrow ablated the chemoprotective effect without altering proliferation. Inhibiting these integrins resulted in substantially fewer bone metastases (but was not totally preventative), providing a strategy to disrupt this protection [101].

Other bone marrow niche components that regulate tumor dormancy—Neural components of the bone marrow regulate HSC quiescence and may regulate DTCs in parallel, including pro-quiescent sympathetic nerve fibers and nonmyelinating Schwann cells [48, 60, 102]. Conversely, norepinephrine induced prostate DTC proliferation as well as downregulated the pro-quiescent factor GAS6 [103]. Chronic stress act' vat's the sympathetic nervous system to induce RANKL signaling in osteoblasts, promoting breast cancer migration to the bone [104]. These studies highlight the neural response to stress that can, in turn, affect metastasis.

Adipocytes fill the spongy marrow of the central cavity, stratified into adipocyte-rich yellow marrow and adipocyte-poor red marrow. Red marrow is enriched in HSC activity, whereas yellow marrow suppresses hematopoiesis through pro-quiescent cytokines like adiponectin [105, 106], leading to the question of how adipocytes influence DTCs. Adipocyte-rich environments inhibited T-cell acute lymphoblastic leukemia (T-ALL) proliferation, resulting in slow-cycling, metabolically-altered, chemoresistant DTCs [107], and chemoprotected multiple myeloma cells via induction of anti-apoptotic autophagy [108]. Future studies should assess the effect of adipocytes on solid tumor DTCs.

Acellular environmental facers also play prominent roles in the bone marrow dormancy niche. Fibrillar fibronectin associated with urokinase plasminogen activator receptor suppresses p38 and de-represses ERK1/2 in the human squamous cell carcinoma HEp3 line [109]. The result is akin to simultaneously accelerating and lifting the breaks off dormant DTC growth. Environmental retinoic acid (RA) maintains HSC stemness [110], so it is no surprise that RA promotes DTC survival and cell cycle arrest through the orphan receptor NR2F1 and transcription factor NANOG [111]. This mechanism is not bone marrow-specific, as RA promotes quiescence in other tissues like the lung [111].

Immune regulation of bone dormancy—The bone marrow is a critical immune organ, organizing an immune cell network and coordinating B cell production [112]. DTC^{+} bone marrow from breast cancer patients is depleted in $CD3^{+}$ T cells, yet enriched for tumor antigen-specific T cells [113]. The persistence of DTCs in such an environment suggests immune evasion, possibly through downregulation of MHCI [114] or regulatory T cell (Treg)-induced immune-suppression [115]. Interferon gamma (IFNγ) treatment may enhance DTC antigen presentation, boosting endogenous immune activity [116]. IFNγ

also directly induced DTC dormancy through antiproliferative STAT1 signaling [117] and cyclin suppression [118], mirroring $CD8^+$ T cell IFNγ induction of B cell leukemia cell cycle arrest [119]. IFN response genes were lost in proliferating bone-derived prostate cancer DTCs compared to those derived from the lung, and suppressing Type I IFN signals activated dormant RM1 prostate cancer DTCs to generate metastases in the bone [120]. Collectively, these data point to IFN signaling as an immune-derived mediator of dormancy in bone marrow. Perivascular MSCs alter the immune landscape for DTCs by inhibiting $CD4^+$ and $CD8^+$ T cell proliferation [121] and enhancing Treg and Th2 $CD4^+$ T cell proliferation [122], generating an immunosuppressive environment. $CD150^{hi}$Tregs also play a non-immunological role in promoting HSC quiescence and engraftment via the production of extracellular adenosine, which protects DTCs from oxidative stress [123]; this protective role may be relevant to bone marrow DTCs as well.

1.4 Activation of quiescent DTCs in the bone

Activation of quiescent DTCs can be attributed to loss of the quiescent niche. Physiologic bone remodeling provides an illustrative example. The endosteum experiences local upheaval where bone is resorbed and remodeled. Osteoclast-driven resorption sites are rare, representing 1% of the endosteum, while osteoblast-driven remodeling of the tone occurs more frequently, across approximately 20% of the endosteum at a given time [45, 46, 124]. During remodeling, RANKL-activated osteoclasts secrete proteolytic enzymes that dissolve the bone matrix, releasing sequestered growth factors such as TGF-β1 (Fig. 1). These enzymes also cleave endosteal factors that maintain HSC and/or DTC quiescence including SCF, CXCL12, and osteopontin [125]. Decreasing oxygen levels and proton release result in an acidified, hypoxic environment [126] that can induce DTC proliferation directly or through angiogenesis [66, 93]. The capillary network directly above remodeling sites increases in density, enhancing trafficking of stromal cells and potentially DTCs as well as undergoing angiogenesis [124]. Endothelial sprouting induces proliferation of dormant MDA-MB-231 breast cancer DTCs through loss of TSP-1 and increased TGF-β1 and periostin (POSTN) in the niche [66]. This is supported by the observation that perivascular cells around remodeling endosteal capillaries increase proliferation [124], alluding to consequences for dormant perivascular DTCs.

The dynamic resorption environment directly activates quiescent myeloma DTCs [19], although the relatively low incidence of remodeling sites may explain the disparity between the frequency of bone DTCs and actual colonization and lesion formation. Activating osteoclasts led to fewer DTC niches and non-dividing myeloma DTCs, but curiously did not correlate with increased overall tumor burden [19]. A two-hit strategy of disturbing the quiescent niche and blocking the immune response would help parse out the source of DTC elimination. Increasing osteoclast activity and recruitment through enhanced VCAM-1 expression was also sufficient to induce dormant mammary DTC activation [127], supporting the role of osteoclasts in dormancy reversal.

The bone marrow is susceptible to changes due to fluctuating hormones during menopause, which is especially relevant for ER^+ breast cancer DTCs. In a modified bone marrow microvascular niche model testing several breast cancer cell lines including MCF7, BT474,

MDA-MB-361, and MDA-MB-231, loss of estrogen induced angiopoietin-2 expression by the bone marrow niche, antagonizing endothelial receptor Tie2 and TSP-1 signaling and leading to ER^+ breast cancer cell growth [128]. Estrogen also negatively regulates IL-6 levels, which drive early breast cancer DTC progression [24]. During menopause, estrogen decreases and serum levels of IL-6 increase up to 10-fold [129–131], which may activate quiescent DTCs in postmenopausal women. Hormone replacement therapy counters rising IL-6 levels [129], potentially slowing DTC progression. Given the effects of estrogen loss, menopause may be a key point to anticipate relapse of residual bone marrow disease.

1.5 Therapeutic outlook

Adjuvant therapies against bone metastases have been successful particularly in breast (50% response) and prostate (70–80% response) cancer [132], but the treatment of dormant DTCs in bone is largely unaddressed. G-CSF mobilizes prostate cancer DTCs from supportive niches [133] and may chemosensitize DTCs when used prior to treatment; conversely, quiescence could be enforced by antagonizing bone resorption. Treatments such as the bisphosphonate zoledronic acid and anti-sclerostin antibodies counter bone resorption by killing osteoclasts [134, 135]; such an approach would preserve quiescent niches to maintain DTC dormancy. However, pharmacological inhibition of niche remodeling was behind the development of anti-RANKL therapies, targeting a pivotal signaling node associated with osteolytic bone degradation and metastasis [136]. Although anti-RANKL treatment benefitted lung cancer patients [137] and resulted in fewer fractures for postmenopausal breast cancer patients [138], it did not extend disease-free or bone metastasis-free survival in a large five-year breast cancer clinical trial [139]. Therefore, more specific approaches to target the DTC niche and deprive DTCs of pro-survival cues are urgently needed.

2. Lung

2.1 Metastasis to the lung

Breast and colorectal cancer have the highest incidence of metastasis to the lung. In a SEER meta-analysis, 36.4% of metastatic breast cancer patients [140] and 32.9% of metastatic colorectal patients [141] develop lung metastasis, which autopsies indicate could be as high as 71% of metastatic breast cancer patients [142]. Although only 3.6% of metastatic prostate cancer have pulmonary metastasis [143], hormone therapies may enhance this rate through transdifferentiation of the tumor into the androgen therapy resistant neuroendocrine subtype [144, 145], which is more likely to generate visceral metastases [146, 147].

Despite the frequency of lung metastasis, isolated lung metastases without a secondary metastatic site are rare across cancers [148, 149], suggesting that lung metastasis is driven by accessibility and permissiveness rather than site specificity. Nevertheless, the lungs are frequently studied in tumor dormancy, with ECM organization and immune influx as major contributors to dormancy regulation.

2.2 The lung microenvironment

Human lungs are comprised of five lobes, three on the right and two on the left side of the thoracic cavity. Each lobe contains branching units of alveoli that facilitate gas

exchange with the adjacent capillary network. The small capillary diameter may slow circulating tumor cells to aid extravasation. The alveoli are lined by epithelial sheets line and surrounded by stroma including pericytes, fibroblasts, and mesenchymal stem cells (Fig. 2). These cells contribute to the epithelial and endothelial basement membrane of the lungs, provide contractile support, maintain the vasculature, and attract migrating immune cells [150]. The ECM of the lung is primarily composed of collagens, laminin, elastin, and glycosaminoglycans, which contribute to alveolar elasticity [151, 152]. Lung metastases tend to be concentrated in the basal and peripheral regions of the lobes due to their increased vascular density and, presumably, coincident frequency of DTC extravasation [153].

2.3 Mechanisms of DTC dormancy in the lung

The lungs are especially prone to metastasis, so it is of great interest to understand tumor progression at this site. Serially passaging breast cancer cells through the lungs generated a lung metastasis signature of 54 genes associated with relapse including chemokines, ECM remodeling proteins, cell adhesion, and transcriptions factors [154]. VCAM-1 was enriched on a lung-metastatic subline of MDA-MB-231 mammary tumor DTCs, enabling newly extravasated DTCs to survive by binding macrophage integrin α4 and activating the PI3K/Akt survival pathway [155]. VCAM-1 expression may be a selection force for pulmonary DTCs since the lungs are leukocyte-rich.

BMP signaling, implicated in bone dormancy [84], is even more active in the lung [156]. BMP4 produced by lung stroma suppressed 4T07 mammary tumor cell proliferation through inhibition of stem cell genes Nanog, Sox2, an Oct4 and induction of metastasis suppressors KIAA1199 and NDRG1 [156]. BMPs :s antagonized by the secreted protein Coco. Knocking down Coco imposed dormancy on highly metastatic 4T1 mammary tumor cells in the lung, but did not prevent metastases to brain, bone, or thyroid. Thus, BMP4 regulates lung-specific dormancy in opposition to Coco.

In lung, the perivascular niche is again implicated in promoting DTC dormancy through endothelial TSP-1, where it is also involved in vessel stability [157] and differentiation of alveolar epithelial cells 45e]. Spontaneous and experimental models of lung metastasis implicated endothelial TSP-1 as a quiescence factor for murine MDA-MB-231 and human T4–2 malignant breast epithelial cells [66].

The lung epithelia form a newly described DTC niche and stimulate D2.0R mammary carcinoma expression of SFRP2, which mediates integrin and survival pathways in a Src-dependent manner [159] (Fig. 2). $SFRP2^{+}$ DTCs deposit fibrillar fibronectin and form cell protrusions to engage with the matrix, coinciding with increased lung metastasis. Although the epithelial signal that induces tumor cell SFRP2 was not identified, these results highlight integrin-ECM interactions as key mediators of lung DTC survival. Fibrillar fibronectin and rho kinase have also been tied to quiescence in a cell culture model of breast cancer, strengthening the association between matrix organization and dormancy [160].

Immune regulation of lung dormancy—Studies suggest that lung DTCs are constrained by the adaptive immune system [161, 162], and slow-cycling breast and lung carcinoma cells derived from patient tumors downregulate ligands for natural killer group

2 member D (NKG2D) receptors to evade NK cell detection [163]. $CD8^+$ T cell depletion allowed emergence of previously undetectable DTCs in the lungs in fibrosarcoma [164], melanoma [162], and mammary carcinoma [165, 166] experimental and transgenic tumor models, while $CD4^+$ T cell depletion only partially suppressed fibrosarcoma metastasis [164]. Intriguingly, suppression of melanoma and mammary carcinoma metastasis was tied to a cytostatic effect of $CD8^+$ T cells [162, 165]. Metastatic outgrowth could be phenocopied by adoptive transfer of pro-tumorigenic MDSCs, which suppress CD8+ T cell activity [166]. While it is unclear whether $CD8^+$ T cells directly suppress cell cycle engagement or select for non-proliferating cells through immune editing, these studies collectively demonstrate how adaptive immunity actively suppresses DTC awakening in the lung.

2.4 Activation of quiescent DTCs in the lung

As an air-tissue interface, the lungs are on the frontlines of environmental and pathological insult. Inflammation awakens dormant mammary carcinoma D2.0R lung DTCs by directly influencing the DTCs as well as modulating the stroma and immune milieu, indicating that therapeutically targeting inflammation may be particularly effective in this tissue [15].

The lung ECM is dominated by type I and II collagens, with additional type III collagen and elastin in the alveolar interstitium [151, 152]. The ECM organization becomes altered through environmental insult. Lung fibrosis dominated by collagen I (col-1) enabled integrin β1-mediated outgrowth of dormant D2.0R cells through phosphorylation of Src, FAK, myosin light chain kinase, and actin stress fiber formation [91, 167]. Fibrillar col-1 also promoted metastatic colonization through binding discoidin domain receptor 1 (DDR1) on the tumor cell membrane, subsequently activating PKC/JAK2/STAT3 signaling via syntenin 2 and tetraspanin TM4SF1 [92]. Fibrillar fibronectin led. to dormancy awakening of SCC HEp3 cells through independent but synergistic inhibition of cell cycle repressor p38 and de-repression of ERK1/2 [109]. While lung DTCs were not as dormant as those in the bone marrow, p38 was required for short-term SCC [109] and colon cancer dormancy in the lung [168]. Concordantly, MMP-2 degradation of a fibrillar fibronectin matrix led to outgrowth of several human breast cancer cell lines in culture [160], further pointing to the importance of ECM architecture to suppress DTC outgrowth.

As the mechanisms underlying the fibrotic awakening response are better understood, it is critical to identify the triggers of fibrosis. Chronic inflammation is a well-known inducer of systemic stress, which can lead to fibrosis. But recently, it was shown that even short, sustained inflammation from either bacterial lipopolysaccharide (LPS) or cigarette smoke can modulate a suppressive niche into a growth permissive one via the immune system. Brief exposure to LPS or cigarette smoke activated release of neutrophil extracellular traps (NETs) and proteolytic enzymes that cleave laminin-111, liberating a peptide that activates dormant D2.0R DTCs, and quiescence factor TSP-1, thus permitting DTC outgrowth [16, 169] (Fig. 2). Fibrosis may also be induced after surgery through lysyl oxidase (LOX)-mediated collagen crosslinking, which allows metastatic outgrowth of undetectable DTCs in lung [170]. Surgery plays a secondary role in activating T cell-dependent lung metastasis [15]. The effect could be abrogated with anti-inflammatory treatment, linking inflammation to adaptive immunity and tumor progression. This phenomenon appears to hold up in

patients [171], offering an inexpensive intervention to counter inflammation and prevent DTC emergence.

Inflammation directly induces epithelial-to-mesenchymal (EMT) transition of dormant DTCs from quiescent, differentiated epithelia to an invasive, dedifferentiated mesenchymal phenotype. Different environmental stressors converge on an EMT response, imbuing dormant DTCs with metastatic capability. LPS induces EMT in a latent subline of mammary carcinoma D2A1 cells through Zeb1 expression, greatly enhancing metastasis in the lung [17]. Hypoxia also induces EMT of DTCs through upregulation of lysyl oxidase-like 2 (LOXL2) [172]. Canonically a crosslinking enzyme [173], LOXL2 initiates EMT and a stem cell-like phenotype in dormant mammary DTCs, resulting in lung metastasis [172].

Finally, inflammation has been linked to dormancy awakening through angiogenesis and concomitant loss of perivascular quiescence factors and gain of pro-metastatic factors like tenascin-C (TNC) and POSTN [66, 174]. Adult angiogenesis occurs during wound healing, hypoxia, or under oxidative stress, which is especially relevant to the lungs. Notably, TNC secreted by lung DTCs themselves promotes survival and invasiveness through Notch/Wnt signaling [175]. DTCs within the lung also instruct the stroma to produce POSTN, which further promotes metastasis [176]. Thus, under inflammatory conditions, dormant DTCs in the lung receive autocrine and paracrine growth cues, ultimately leading to metastatic awakening.

3. Brain

3.1 Metastasis to the brain

Brain metastases are detected in 8.5–20% of metastatic patients, most commonly in lung, renal, breast, melanoma, and colorectal cancer [177–180]. Autopsy studies indicate the incidence may be much higher, with up to 30% of breast cancer patients exhibiting metastatic lesions [180, 181]. The incidence of brain metastasis seems to be increasing due to more accurate imaging technologies and systemic therapies that extend patient survival, such as trastuzumab for HER-2^+ breast cancer or taxanes for ovarian cancer [179, 182, 183] (although not all studies agree [177]). The increasing incidence of brain metastasis highlights a critical need to understand the niche(s) that enable DTC survival better so we can identify vulnerabilities and prevent metastasis.

Brain metastases tend to emerge at watershed areas between vascular beds [184]. These regions are supplied by the smallest arteriolar branches, suggesting that vessel diameter aids DTC extravasation. The cerebellum is an overrepresented target for breast and gastrointestinal metastases and the frontal lobe for melanoma metastases when normalized for lobe volume [185], suggesting different metastatic potentials between the different lobes. The distribution of DTCs in different mouse models varies (e.g., brain-tropic MDA-MB-231 breast cancer cells are enriched in the frontal and parietal cortex following intracardiac injection) [186], indicating additional species-specific differences that may necessitate new models to understand the differences observed in humans.

3.2 The brain microenvironment

The brain is a dense network of neurons, glia, and other stromal cells. The immune compartment consists of resident macrophages (microglia) as well as adaptive immune cells and natural killer cells at homeostasis and in disease states such as acute viral infection [187–194]. Fibrous ECM components like fibronectin and collagen are nearly absent from the adult brain, whereas proteoglycans fill the intercellular space and support synaptic contacts [195–197]. Endothelia bearing tight junctions, smooth muscle cells, pericytes, astrocyte endfeet, and neural synapses form a physical and molecular seal between solutes and the central nervous space called the blood-brain barrier (BBB) (Fig. 3). The basement membrane (BM) subtly changes between the vasculature and the outer side of the parenchyma (e.g., in laminin composition) [198–200]. Lymphatic vessels, only recently observed in the superficial dura mater, drain excess interstitial fluids but do not penetrate into the gray matter [201, 202].

3.3 Mechanisms of DTC dormancy and activation in the brain

Research on cellular dormancy of DTCs in the brain is not as well-established as in other organs. Few studies have attempted to model brain DTC dormancy, with notable exceptions [203, 204], but delayed relapse data [177], primary glioma research [205–207], and intravital imaging [208, 209] inform our understanding of the brain dormancy niche.

Tumor cell dissemination to the brain is not uncommon [210] but nevertheless is strenuous for DTCs [208, 209, 211]. Certain molecules are required to cross the BBB including the adhesion protein ST6GALNAC5 [212], proteolytic enzyme cathepsin-S [213], chemokine receptor CCR4 [214], and MMP9 to remodel the stroma [209]. Tumor-secreted exosomes containing cell migration-inducing and hyaluranon-binding protein (CEMIP) enhanced brain invasion and vascular interactions [215]. Fittingly, DTC extravasation to the brain takes longer than other tissues [216]. Once in the brain, the next major bottleneck for DTCs is overcoming microenvironmental barriers to growth. The emergence of brain metastasis only after chemotherapy and frequency of delayed relapse of a year or more after initial diagnosis (~28%, 66/232 cancer patients with brain metastasis) [177] suggests two interesting points: 1) that brain DTCs need time to overcome these barriers to growth, and 2) that DTCs are protected from chemotherapy.

Intravital imaging highlights the critical role of blood vessels in DTC survival. The endothelia are the first cell type that an extravasating DTC encounters, and failure to generate appropriate contacts with vessels leads to cell death for human melanoma, lung, and breast cancer DTCs [203, 208, 217]. Within this niche, some tumor cells (e.g., melanoma) are highly motile whereas others (lung carcinoma) remain static [208]. DTCs specifically engage in distinct ‘spreading’ over the vessels via the cell adhesion molecule L1 (L1CAM) (Fig 3.), which displaces vessel pericytes and promotes colonization through the mechanically-activated Yes-associated protein (YAP) and myocardin-related transcription factor (MTRF) [218]. VEGF-A blockade prevented PC14-PE6 lung carcinoma DTCs from progressing (ostensibly by stabilizing the vessels [66]) and pushed DTCs to adapt to different vessel interactions [208], highlighting a functional dependency on the vasculature. Brain endothelia provide chemoprotection to murine mammary tumor cells in culture

through gap junction communication and secreted endothelin, resulting in 50% reduction of tumor cell death with taxol treatment [219]. When inflamed by tumor-secreted CEMIP$^+$ exosomes, the endothelia commence vascular remodeling that promotes metastasis [215].

After emerging on the abluminal side of the vasculature, brain DTCs encounter glial and mural cells that support the vessels. Astrocytes are a significant cellular component of the brain parenchyme. At homeostasis, astrocytes support the integrity of the BBB through endfoot contact with the vessels and expression of Fas ligand, which repels infiltrating lymphocytes [220]. But in response to inflammation, metastasis, or glioma-secreted exosomes, astrocytes undergo reactive gliosis and become pro-metastatic [186, 209, 221–223]. Brain metastatic breast and lung carcinoma cells directly transfer cGAMP to astrocytes through gap junctions which activates astrocytes to secrete paracrine IFNα and TNF back to the tumor cells, promoting survival and chemoprotection through STAT1 and NF-κB pathways [224, 225]. Reactive astrocyte gap junctions also chemoprotect MDA-MB-231 breast and H226 lung cancer DTCs through endothelin-IL-6/IL-8 signaling [219], and sequester intracellular calcium from human melanoma cells [226]. Reactive astrocytes suppress DNA methyltransferase 1 (DNMT1) in human melanoma, breast, and lung DTCs through an unknown secreted factor, which activates pro-survival L1CAM and the anti-apoptotic molecular chaperone αB-crystallin (CRYAB) [204]. Astrocytes promote metastatic growth through secreted IL-6 and TNF-α [227] and S100A under systemic estrogen [228]. Strikingly, brain metastatic MLA-MB-231 breast cancer DTCs encouraged neural progenitor cell differentiation into astrocytes via BMP2, fine-tuning their niche into a permissive environment [229].

However, reactive astrocytes can also prevent metastasis through cytostatic functions. They produce plasminogen activator (PA), which liberates paracrine FasL to kill invading DTCs, and inhibit L1CAM preventing spreading and vascular co-option [217]. Brain-metastatic H2030 lung carcinoma and MDA-MB-231 breast carcinoma DTCs neutralize PA through serpin expression, which canonically protects neurons from elimination [217].

The chemical composition of the brain presents an additional barrier to DTC growth. The brain relies on specialized lipids instead of triacylglycerols to support neural activity [230], generating a unique lipid microenvironment to which brain DTCs must metabolically adapt. Using a panel of 50v human cancer cell lines across 21 different tumor types, two metabolic dependence have been uncovered: upregulation of SREBF1, a transcription factor that alters DTC fatty acid metabolism and enables proliferation of micrometastases, and engaging the fatty acid transporter CD36 and fatty acid-binding protein FABP6 to acquire lipids [231]. These adaptations demonstrate DTC plasticity to meet the unique demands of the brain microenvironment.

Immune regulation of brain dormancy—Brain DTC-immune cell interactions are similarly context-dependent. Microglia accumulate in or near metastases [60, 209, 232, 233]. They are canonically cytotoxic through secretion of nitric oxide [234] but assist human MCF7 breast and murine M410.4 mammary carcinoma DTC invasion through Wnt signaling [235] and MMP3 activation [236]. Contrary to most organs, inflammation *reduced* the pro-invasive effect of microglia, underscoring brain-specific protection from

excessive inflammation [235]. Microglia-conditioned media upregulated quiescence and survival factors including TSP-1, BCL6, SMAD3, LIF, L1CAM [205] and GAS1 [206] in glioma organoid spheres, and it would be interesting to assess their effect on metastasizing cell types. Samples derived from human patients with breast or lung cancer metastasis to the brain revealed that NK cells actively survey for DTCs, which shed NKG2D ligands upon quiescence [237] and express them with re-entry into the cell cycle, resulting in elimination by NK cells [218].

As more barriers to growth in the brain are elucidated, we will gain a better understanding of the dormancy niche, and the diverse ways DTCs must adapt to co-opt the niche to survive (Fig 3). Future studies should consider the role of adaptive immunity [188, 238], which will be aided by advancing technologies and pre-clinical models of metals, *en route* to therapeutic approaches for this devastating aspect of metastasis.

4. Liver

4.1 Metastasis to the liver

The liver is an enzyme-rich organ that processes metabolites, synthesizes bile, and maintains blood glucose levels. It is the most common site of distant metastases, excluding lymph nodes [239, 240]. Across metastatic patients, liver metastases most frequently occur in pancreatic (85%), colorectal (78%), and esophageal cancers (52%), as well as breast (30%) and lung (16%) cancer [241]. Treatment option for liver metastasis are limited. Surgical resection is considered the only curative therapy [242, 243] and is standard of care for pancreatic and colorectal cancer metastasis. However, the benefits of surgery are modest and may induce liver injury, which in turn exacerbates metastasis [244–247]. The benefit of hepatic resection in other cancer types such as breast is promising [248], correlating with a 5-year survival rate of 22–41% [249, 250], yet only a subset of patients qualify for surgery [242]. Thus, better treatment options are crucially needed to manage metastatic liver disease.

Tumor dormancy in the liver is supported by autopsy and clinical samples [210, 251, 252] as well as experimental models [21, 252–255]. Mammary carcinoma DTCs persist as single cells in the liver for 11 weeks post-injection and reinitiate growth upon *ex vivo* isolation [21]. DTCs access the liver with relative ease through low-pressure, fenestrated sinusoids [256] and intravital imaging confirms that the liver is more prone to DTC extravasation than the lungs [257]. The liver also exhibits high retention rate of tumor cells: meta-analyses determined that the liver is the most frequent secondary site for metastases, and, conversely, primary liver cancer metastasizes the least compared to other primary tumor types [239, 240]. This paints a picture of the liver as a “sink” from which primary tumors or secondary metastases are less likely to spread. Although the steps of metastatic liver colonization have been described [258], investigating interactions between DTCs and the liver niche particularly during dormancy would illuminate the liver niche’s capacity for supporting dormancy.

4.2 The liver microenvironment

Macro- and micro-levels of organization foster liver cell specialization and function [259, 260]. Epithelial sheets of hepatocytes, the parenchymal cell that comprises the bulk of the liver mass and functions in metabolism, storage, and bile secretion, converge on central portal tracts made up of a triad of vessels: a portal vein, artery, and bile duct (Fig. 4). The portal triad is orthogonally connected to an efferent central vein via fenestrated sinusoids that run between the hepatocyte sheets. The sinusoids are comprised of liver sinusoidal endothelial cells (LSECs) and lack a typical organized basement membrane [261]. 70–80% of the liver's blood supply passes through the low pressure portal vein and the remaining 20–30% comes from the portal artery [256, 262]. These blood supplies intermix as they flow across the sinusoids, another low-pressure system where Kupffer cells, the resident macrophage, reside. The subendothelial Space of Disse drains lymph and contains hepatic stellate cells (HSteCs) that store fat-soluble metabolites like retinoids and produce the majority of ECM fibers in a loose subendothelial basement membrane [263]. HSteCs also have contractile function and regulate sinusoidal blood flow through LSEC-generated nitric oxide and endothelin signaling [264].

Single-cell analysis of the liver revealed stromal subpopulatioi.s and specializations across the hepatic zones [259], which radiate outward from the portal triad to the central vein following an oxygen and nutrient gradient [265] (Fig. 4). Zone 1, closest to the portal triad, experiences approximately twice the oxygen tension and, higher levels of ROS versus Zone 3, closest to the central vein [266]. Gluconeogenesis and ureagenesis occur in Zone 1, whereas Zone 3 is glycolytic and the site for glutamine synthesis [265]. ECM composition also changes between zones, with Zone 1 rich in type I and III collagens and Zone 3 in collagens IV and VI [267]. LSECs in Zone 3 undergo a greater fibrotic response to cirrhotic injury compared to Zone 1 LSECs, revealing differential vulnerability or activation thresholds across the zones [268]. How zonation impacts tumor dormancy is unknown.

4.3 Mechanisms of DTC dormancy in the liver

The liver imposes a strong quiescent phenotype on DTCs, as liver-derived mammary DTCs required two to three times as long to resume proliferation *ex vivo* compared to DTCs derived from the lung [253]. Patient autopsies revealed single tumor cells scattered throughout the liver that had not progressed to metastasis [210]. Additionally, primary hepatocellular carcinoma undergoes reversible quiescence and differentiation into normal stroma upon MYC inactivation [269]. Together, these studies indicate the liver microenvironment resists metastatic growth, but few studies have directly addressed the liver dormancy niche. Here, we examine known mechanisms of quiescence and potential dormancy niches to aid in this challenge.

Metabolism is a primary function of the liver and DTCs need to adapt accordingly in order to survive and grow. Inability to resolve endoplasmic reticulum (ER) stress in pancreatic ductal adenocarcinoma mM1DTLB DTCs led to quiescence and, critically, MHCI downregulation, which could be restored through pharmacological relief of ER stress or rescuing unfolded protein response machinery [252]. The immune system plays a key role in selecting for quiescent $MHCI^{-}$ DTCs: in T cell-depleted mice, quiescent DTCs reverted

to an MHCI$^+$ proliferative phenotype, whereas few DTCs or metastases could be detected in an immune-competent setting [252]. DTCs did not express NKG2D ligands required for NK recognition, so T cells are likely the dominant immune cell. Indeed, a 'wave of apoptosis' has been described in mouse models of hepatic mammary carcinoma metastasis as the immune system eliminates lesions [21]. Although immune recognition can be enhanced through relieving ER stress, this carries the risk of awakening quiescent DTCs. Alternatively, treatment with an ER stressor (tunicamycin) drove DTCs into quiescence, suggesting a strategy for maintaining dormancy if toxicities can be minimized [252]. Thus, adapting to the liver requires a trade-off between metabolic adaptation and immune evasion.

Although the preferential dormancy niche(s) for liver DTCs is unknown, the perisinusoidal space which DTCs encounter upon extravasation is rich in fibrillar collagens type III, and V, non-fibrillar collagens type IV and XVIII, as well as basement membrane components including fibronectin, perlecan, and laminins [267, 270, 271]. Type IV collagen promotes both survival and metastasis for liver DTCs [272, 273], and laminins and perlecan are noted quiescence promoters in other tissue sites [274, 275]. *Ex vivo* imaging confirms that vessel association is critical for extravasated DTC survival [257], but must be examined thoroughly in preclinical models.

Parenchymal hepatocytes induce mesenchymal-to-epithelial transition (MET) and quiescence in prostate cancer cells via p38 and ERK1/2 n a co-culture model [276]. Prostate and breast cancer models confirm that hepatocytes are critical for DTC quiescence and proliferation-independent chemoresistance [277–279]. Further, breast cancer DTCs adopt an intriguing hepatocyte-like 'replacement' organization in human patients [280], suggesting that communication with the parenchyma is beneficial for DTCs. Co-culture studies indicate that hepatocytes are the primary effector^ of tumor quiescence compared to NPCs [281]; however, growth on plastic activates HSteCs and phenocopies inflammation [282], which could confoundingly enhance tumor outgrow *h. Thus, it is likely that both hepatocytes and NPCs contribute to the dormant DTC phenotype in healthy, homeostatic tissue, which is lost upon stromal activation in response to injury.

Non-parenchyma cells (NPCs) including LSECs, Kupffer cells, and HSteCs support trafficking and quiescence of resident liver cells, and it is conceivable that these mechanisms impact DTCs. For example, LSECs secrete CXCL12, which attracts CXCR4$^+$ DTCs [283], and nitric oxide, which promotes HSteC quiescence and contractility [284]. HSteCs promote vessel integrity through VEGF and nitric oxide [285], stabilizing a prospective quiescent niche.

4.4 Activation of quiescent DTCs in the liver

The quiescent hepatic microenvironment resists metastasis, which can be reversed upon stromal activation [286]. Two major sources of physiological disruption within the liver, fibrosis and regeneration after injury, are associated with activation [287–290] and may be key turning points in the dormancy timeline that promote awakening.

Liver fibrosis results from stromal cell activation following injury. It has numerous etiologies such as alcohol abuse, hepatitis, fatty liver disease, or surgery which converge

on a state of inflammation [288, 291]. HSteCs are the primary effector of fibrosis, and activate from quiescent to proliferative hepatic myofibroblasts (HMFs), upregulate αSMA, lose their retinoid stores, and increase deposition of ECM fibers, especially collagens [292]. TGF-β1 [293, 294], PDGF [295], FGF [296], and ECM stiffness [297] contribute to the HSteC activation process, creating a pro-fibrotic feedback loop. The ECM stiffens as HSteCs deposit excessive collagen (predominantly collagens I, III, and IV [263, 298]), tissue inhibitor matrix metalloproteinase enzymes which antagonize MMPs to prevent matrix degradation, and LOX, which crosslinks collagen further [299, 300]. The liver sinusoids undergo capillarization [301] and hepatocytes lose their microvilli [302]. Proteins that are not normally a component of the healthy liver ECM appear, such as pro-metastatic TNC and MMP9 [303, 304].

Increased matrix stiffness [254, 305] and HMFs [286, 306] are highly associated with metastatic outgrowth, and potentially a source of awakening signals for dormant DTCs. HMFs secrete proliferative cytokines including RANTES [307] which may act on DTCs and inhibit T cell activity through B7-H1/PD-L1 ligation [308, 309]. In culture, MCF7 and MDA-MB-231 breast cancer cells treated with conditioned media from activated HSteCs exhibited increased proliferation when compared to conditioned media from quiescent HSteCs [306]. Thus, activated HSteCs promote DTC outgrowth and metastasis by altering the ECM, direct proliferative signaling, and immune suppression. Targeting the underlying causes of fibrosis [310–312] may be a viable strategy for maintain tumor dormancy [313]. A key aspect is restoring HSteC quiescence, which has been achieved through all-trans retinoic acid [286], vitamin D [314], and exposure to LSEC-generated nitric oxide [284]. It is also relevant to note that NK cells counter fibrosis by eliminating activated HSteCs with low MHCI expression [315, 316], and restrain activated hepatocyte proliferation in a IFN-γ-dependent manner [317]. It is possible that the anti-fibrotic role of NK cells helps sustain DTC quiescence, although a direct relationship between NK cells and DTC dormancy has yet to be established.

Liver regeneration is the controlled healing response to tissue injury such as surgical resection. Surgery is associated with relapse as a result of inflammation [15, 318, 319], but liver regeneration is unique from the trauma of surgery. During regeneration, the liver stroma undergo rapid yet controlled hyperplasia without incurring inflammation and actually suppress fibrosis [320, 321]. The endothelium may be at the heart of the balance between fibrosis and regeneration, as the angiocrine response to injury hinges on the dominance of CXCR7 versus CXCR4 signaling and the sphingosine-1-phosphate receptor [321, 322]. Depending on the balance of signals, activation is transduced from LSECs to HSteCs, cascading into either the fibrotic or regenerative response. When regeneration commences, hepatocytes and NPCs exit G0 phase and undergo mitosis under the regulation of cytokines including TNF-α, NF-κB [323], IL-6 [324], and growth factors [325, 326], while TGF-β and activin terminate stromal proliferation [327]. In this context, a bystander dormant DTC may be exposed to the rich proliferative signals in the regenerative microenvironment and enter the cell cycle. The role of liver regeneration in metastasis is well-studied [245, 290, 328], but the relationship between regeneration and awakening from dormancy has not been elucidated [255]. Since surgical resection is a standard treatment for liver metastasis for

some cancers, it is critical to understand how fibrosis and regeneration impact dormant DTCs to avoid incidental DTC awakening.

In summary, the liver provides a dormancy niche for DTCs, which can be perturbed through fibrosis and injury. More research is needed to investigate hepatic regulators of quiescence and the interactions between DTCs and the stroma. While fibrosis activates the quiescent tissue, preventing inflammation or reversing it may help sustain DTC dormancy [313].

5. Lymph node (LN)

5.1 Metastasis to the LN

The lymph node (LN) is the most frequent site of metastases across tumor and tissue types [240]. Although LNs themselves are not vital organs, LN metastasis strongly associates with distant metastases and is a key prognostic factor in staging practices [329–334]. However, there is some controversy over their significance [335, 336]: Do LN DTCs contribute to distant metastases? Does the ability to disseminate to LNs reflect tumor aggression or the proximity and non-selective nature of LNs? How do the inevitable tumor-immune interactions within the LN influence tumor development at local and distant sites?

While we cannot track the cell of origin for a metastatic lesion within a human patient, experimental evidence has convincingly shown that LN DTCs ca n migrate to subsequent LNs [337] and seed metastases in distant tissues [334, 338–341], via blood or lymph [341–343]. Clinical evidence supports the notion that LN DTCs contribute to disease, especially over a longer duration of follow-up. The strongest example of this has been in breast cancer, where the MIRROR (Micrometastases and Isolated Tumor Cells: Relevant and Robust or Rubbish?) study, NSABP B-32 clinical trial, and meta-analyses have shown that isolated tumor cells in patient LNs independently predict worsened overall and disease-free survival over five to ten years post-diagnosis versus node-negative patients [344–349], and similar trends hold in colon cancer [350]. Further, this may be a conservative measurement given that 13–25% of histologically “node-negative” patients have occult metastases upon closer examination [346, 348, 351, 352]. Cumulative incidence curves comparing breast cancer patients stratified by nodal status reveal a latent phase in the isolated tumor cell ($pN0_{(i+)}$) group that lasts approximately 36 months before the rate of adverse events rapidly increases [348, 353]. While it is not possible to assign a cause for this rapid switch base d on these data, it aligns with the paradigm of tumor dormancy in which single or small clusters of tumor cells reside in a quiescent niche within a tissue in a state of cell cycle arrest until stimulated to divide [354].

Late recurrence in LNs more than 10 years after diagnosis has also been documented for melanoma [355, 356] and gastric cancer [357], supporting the notion that LNs may harbor dormant DTCs for long periods of time. Establishing the role of the LN microenvironment in tumor dormancy is crucial for anticipating late relapse and guiding treatment strategies aimed at preventing distant relapse.

5.2 The LN microenvironment

The lymphatic system is a network of one-way, leaky, fenestrated vessels that collect the interstitial fluid lost in peripheral capillary beds and return it to circulation through the thoracic duct to maintain blood pressure. This fluid, called lymph, carries proteins, cell debris, and immune cells, as well as pathogens and disseminating tumor cells. Intermittent nodules along the lymphatics serve as 'field headquarters' for peripheral immunity, allowing lymph to be surveyed by resident antigen-presenting cells (APCs) and adaptive immune cells in a centralized hub. To facilitate antigen sampling and presentation, LNs are highly compartmentalized, including four major subsets of stromal cells: lymphatic endothelial cells (LEC), blood endothelial cells (BEC), fibroblastic reticular cells (FRC), and contractile pericytes previously referred to as $CD31^{-}$ $podoplanin^{-}$ 'double negative' cells that are not as well-characterized [358, 359] (Fig 5.).

FRCs are the main parenchymal cells that provide structural and functional support to the LN resident cells. They produce and ensheath the conduit network, a series of ECM tubules that facilitates antigen sampling and serves as a scaffold for migrating leukocytes. The tubule core contains collagen I, IV, and XIV and proteoglycans (e.g., decorin, biglycan, and fibromodulin), surrounded by an outer layer of fibrillin, collagen VI, TNC and laminin-332 [359–361]. FRCs also control T cell migration [362] and egress [363], and regulate LN expansion though interactions with active dendritic cells (DCs) [364].

LN LEC form the outer capsule and internal sinuses. When lymph enters via the afferent vessel, it flows into the subcapsular space (SCS) which is separated ii.m the parenchyma by a layer of LEC. Lymph-borne particles are separated by size: small molecules less than 70 kDa or 5.5 nm in diameter can enter the conduit network and flow into the parenchyma, whereas larger particles are confined to the SCS to prevent possible pathogens from accessing the blood [365]. DCs sample small molecules within the conduit system [360, 366] and specialized subcapsular macrophages [367] and DCs [368] sample larger molecules in the SCS.

The outer cortex contains multiple lymphoid lobules consisting of B cells, follicular DCs, and supporting stroma. Lymphatic sinuses interdigitate between the lobules, reaching down to the medulla near the base of the node, which collects lymph from the sinuses into the efferent vessel. T cells reside and activate in the inner paracortex region. Throughout the parenchyma, LN BEC form two distinct vessels: arteries that enter at the hilar base and branch out into smaller capillaries to provide nutrients, and high endothelial venules (HEVs) that enable lymphocyte trafficking through adhesion molecules such as L-selectin and the absence of tight junctions [369]. These subcompartments form very distinct niches, though their respective effect on DTC phenotype is currently unknown.

5.3 Mechanisms of DTC Dormancy in the LN

The LN is a site of carefully coordinated migration and localization, and chemokines help set up the appropriate cues and compartments for immune function. Tumor cells co-opt these signals to enhance LN invasion. Initially, DTCs access the LN through intra- or peritumoral lymphatic vessels [370], as well as the aforementioned blood circulation [338,

339, 341]. Migrating cells entering via the afferent lymphatics express CCR8 which binds to LEC CCL1 to increase motility and facilitate entry into the LN [371]. These signals can be co-opted by DTCs and are required for LN access [372]. Inhibiting CCR8 caused melanoma DTCs to accumulate at the junction of the afferent vessel and LN capsule, conferring a gatekeeping function on CCL1-CCR8 signaling. Migrating DCs and T cells rely on gradients of CCL19, CCL21, and CXCL12 from FRCs and HEVs to transverse the LEC subcapsular floor and migrate into the parenchyma [373–375]. Breast, melanoma, and prostate cancer DTCs also express the cognate receptors CCR7 and CXCR4 [376–378], and inhibition of CXCR4 prevented breast cancer metastasis to the LN [376], suggesting DTCs follow the same cytokines gradients (Fig 5.).

A LN niche for dormant tumor cells has not been defined, but clues can be derived from patient and preclinical models. DTCs in human and animal models tend to concentrate in the SCS, which follows from afferent lymphatic entry [353, 379–385]. For breast cancer, metastases are isolated to the SCS in 47% of involved sentinel LNs, whereas only 3% contain metastatic deposits solely in the parenchyma [385]; for melanoma, 26% of sentinel LN metastasis is subcapsular, with no association found between location of the sentinel LN and microanatomic location of the metastatic deposit [381]. However, microanatomic location of the metastatic deposit predicts non-sentinel LN involvement and malignant outcome. Patients with metastases confined to the superficial SCS of the sentinel LN had an extremely low (statistically zero) probability of further LN involvement [381], indicating that the SCS is cap able of fostering growing metastases but stymies true tissue invasion. Egress through the LN blood vessels or efferent lymphatics would require DTCs to cross the subcapsular floor LECs and transverse the parenchyma, which is possible given that human breast cancer cells can perforate LEC monolayers through the metabolite 12S-HETE [386]. However, SCS containment underscores the notion that not all microanatomic regions of the LN are equally poised to promote metastasis.

Taking a clue from other tissues [66], the LN endothelia may contribute to the dormancy niche. While they come from a common endothelial progenitor [387], LN BEC and LEC are distinct in function and interactions with tumor cells. In one of the few studies addressing LN ECs and DTCs directly, human BEC suppressed MDA-MB-231 breast tumor growth in culture and when co-injected into mice, whereas LEC promoted tumor growth due to secreted EGF and PDGF-BB [388]. Further, LN LEC were conditioned by mammary tumor IL-6 to secrete CCL5 and promote angiogenesis, leading to enhanced LN metastasis [389]. Whether LN BEC suppression of tumor growth is due to a deficit in metastasis-promoting factors or the production of quiescence factors has yet to be determined. Further study would shed light into the functional distinction between the endothelial compartments with respect to tumor dormancy.

To that end, recent single-cell transcriptome profiling of FRC [390], LN BEC (capillaries and HEVs) [391], and LN LEC [392–394] has provided a wealth of insight into specializations and subpopulations of these cells, which may be relevant for tumor dormancy studies. For example, subcapsular ceiling LEC express the machinery to take up modified low-density lipoproteins (LDL) [392], a conserved function with the gut lymphatics [395]. LDL accumulation can disrupt macrophage function, a possible justification for scavenging

LDLs from an immune organ [396], but its impact on LN metastasis and dormancy is not well-understood. LN melanoma DTCs upregulated lipid metabolism pathways to oxidize fatty acid species, and functional inhibition of fatty acid oxidation suppressed LN but not lung metastasis, indicating a unique adaptation for LN metastasis [397]. The effect of fatty acid oxidation inhibition on the immune compartment was not assessed but could hypothetically activate macrophages to eliminate LN metastases. This study opens the door for examining the role of dietary fats in providing fuel for LN DTC proliferation and determining whether LEC LDL scavenging has a protective effect.

5.4 Activation of quiescent DTCs in the LN

While the definition of the LN as a dormancy niche is still under scrutiny, two mechanisms of DTC awakening can be anticipated: the immune suppressive role of LN stromal cells and the dramatic changes that occur with inflammation. Although LN stroma maintain immune function [398], many studies highlight an immunoregulatory role for FRC and LEC [399–401]. Although this role is critical to mitigate autoimmunity, it may hinder defenses against tumor colonization. $CD8^+$ T cells actively constrain LN metastasis [402], but FRC and LEC attenuate T cell effector function through prostaglandins [400], TGF-β1 [400], cyclooxygenase-2 [399], and nitric oxide [403] (Fig. 5).

FRC and LEC engage in direct and cross-presentation of self-antigens, which, in the absence of costimulation, elicits T cell exhaustion through PD-L1 [401, 404–408]. LN LEC cross-presentation of soluble tumor antigens selects against tumor-specific T cells, altering the immune repertoire and leading to enhanced LN metastasis of B16-F10 melanoma cells [409]. LN BEC do not seem to have the same immune-suppressive profile. The consequences of stromal tolerogenesis should be analyzed with respect to both the T cell repertoire and selection of immune-privileged tumor clones in order to clarify its systemic effects.

Inflammation alters the stable environment of DTCs, and has specifically been linked to emergence of D2.0R and latent D2A1 DTCs from dormancy through changes to the microenvironment at other sites [16, 17]. The LN is no exception; in fact, it is an exemplary organ for the study of inflammation-induced stromal changes. Upon recognition of a pathogen, the LN expands up to five-fold over several days with concomitant stromal proliferation and ECM remodeling to accommodate immune influx [410, 411]. After the inflammation is resolved, the LN undergoes shrinkage to report homeostasis. Since stromal stability plays such a prominent role in maintaining DTC quiescence in other organs, the dynamically responsive LN environment may disturb quiescent DTCs and induce awakening.

The initial phase of inflammation is regulated by DCs, and later expansion phases are maintained by B and T cells [412–414]. Upon inflammation, activated DC CLEC-2 binds FRC PDPN which relaxes the FRC network, allowing the LN to expand [364, 415] and resident cells to proliferate [416]. conduit network becomes leaky as FRCs loosen their attachments, exposing ECM components to the parenchyma. FRC expression of ECM components shifts, resulting in downregulation of netrin-1, which has been linked to quiescence factor TSP-1, and upregulation of metastatic niche components such as TNC

and MMPs [359, 417]. DCs and FRCs coordinate secretion of VEGF-A and VEGF-C to induce BEC and LEC proliferation and angiogenesis and lymphangiogenesis [359, 412, 418–420]. As detailed in Sections 1.3 and 2.3, sprouting angiogenesis is associated with loss of the quiescent vascular niche and emergence of activated DTCs in lungs and bone marrow [66], and it remains to be seen whether inflammation-induced endothelial remodeling has a similar effect on LN DTCs. If so, then any kind of inflammatory insult may have drastic ramifications for activating dormant DTCs which could help explain the frequency of LN metastasis. Conversely, treating inflammation through non-steroidal anti-inflammatory drugs may be a viable method to stabilize the stromal architecture and reduce metastatic outgrowth. One might speculate that imposing stromal stability could restrict dissemination from the LN to other sites.

Conclusion

Tumor dormancy is a silent stage of cancer progression that continues to pose a clinical threat. Through a combination of extrinsic forces and ability to adapt, a portion of DTCs co-opt, educate, and evade their way to long-term survival. We are only beginning to appreciate how the unique physiology, architecture, and resident cells of each tissue contribute to the dormancy niche, but it is biologically and clinically compelling to identify common themes and distinct mechanisms of dormancy and awakening.

Vascular association is crucial across all organs discussed in this review. As the first microenvironment encountered by extravasating DTCs, the vasculature pr vides sustaining nutrients and potent pro-quiescence factors. A second theme is co-option of pre-existing niches, such as those that regulate HSCs in the bone marrow, and signaling cascades, such as those that regulate inter- and intra-lymph node trafficking of lymphocytes. Third, DTCs must adapt metabolically, as evidenced in the brain, liver, and lymph node, where fatty acid oxidation is necessary for DTC proliferation. Fourth, the immune system must be evaded or silenced, which DTCs accomplish through downregulation of recognition molecules (e.g., MHCI, NGK2D ligands) or benefitting from immunosuppressive environments. And finally, emergence from dormancy is tied to inflammation across tissues as a result of environmental insult, disease, and surgery, highlighting a common set of condition, that may predict delayed relapse in the clinic. Angiogenesis in response to injury exposes perivascular DTCs to a host of proliferative signals and concurrent loss of quiescence cues. Activated stromal cells release pro-inflammatory cytokines, initiate ECM remodeling, and lose their stabilizing functions. One silver lining may be the utility of treatments that resolve inflammation and restore stromal homeostasis, such as anti-inflammatory drugs and retinoic acid.

We have described known and prospective niches for dormant DTCs in several metastatic tissues, but many unknowns remain, not the least of which is exploring non-metastatic tissues to gain insight into mechanisms of dormancy [421]. As the field pursues these unknowns with rigor, we must deepen our understanding of the complex relationship between tumor cell, niche, and the thorns and rocks which challenge the metastatic process.

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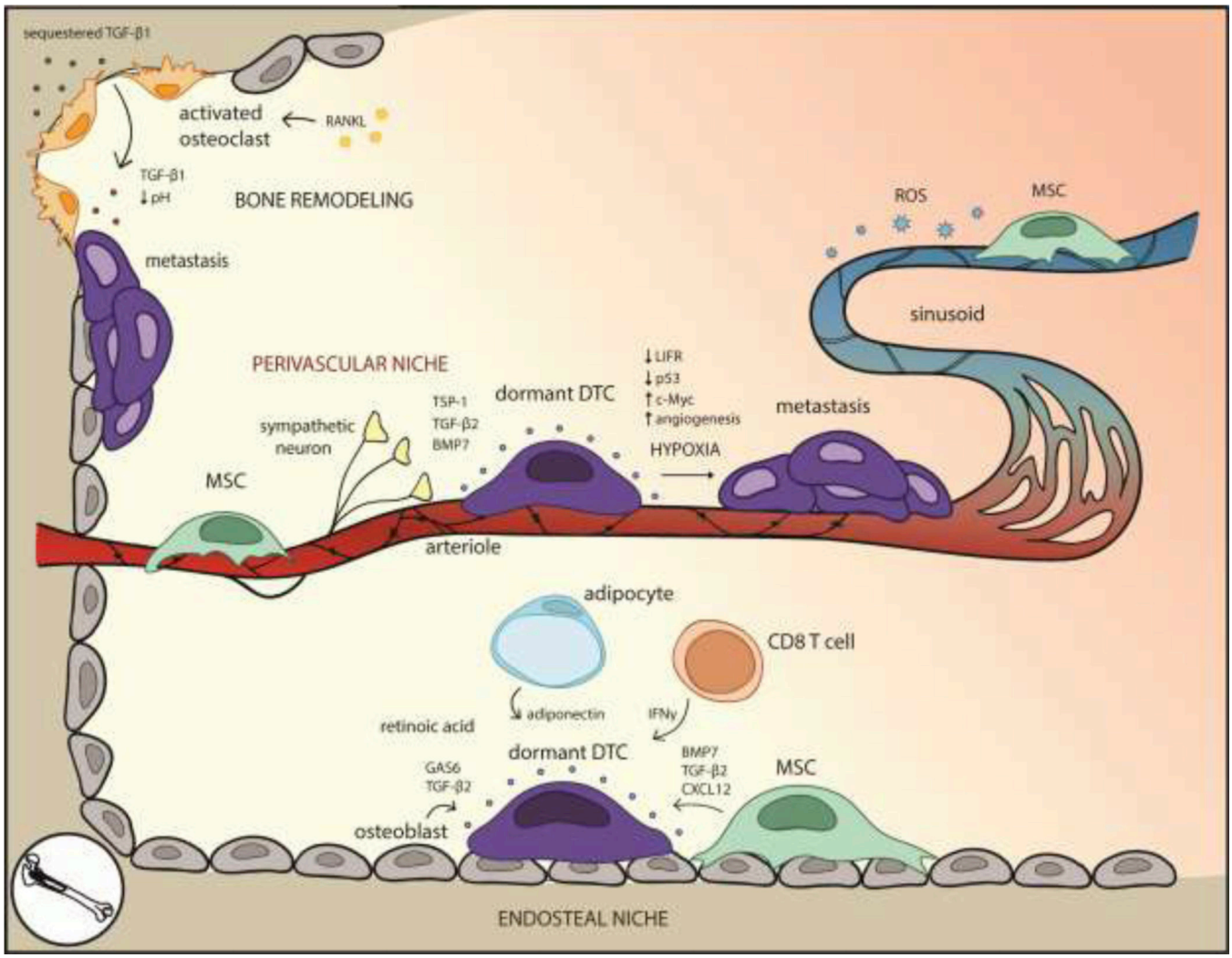

Figure 1. The bone microenvironment for dormant DTCs is well-defined due to established mechanisms that regulate HSC quiescence and bone resorption.

Within the endosteal niche, MSCs, osteoblasts, adipocytes, and $CD8^+$ T cells secrete factors that preserve DTC quiescence. High levels of retinoic acid within the bone marrow induce cell cycle arrest. Awakening can occur as the result of environmental changes brought on by RANKL-activated osteoclasts and bone resorption. In the perivascular niche, arterioles and perivascular cells promote DTC quiescence through basement membrane factors such as TSP-1, although hypoxic regions near the vessels promote angiogenesis, which correlates with metastasis. The leakier sinusoids, by contrast, experience ROS on the basal side of the vessel, possibly inducing DNA damage and stress pathways associated with metastasis.

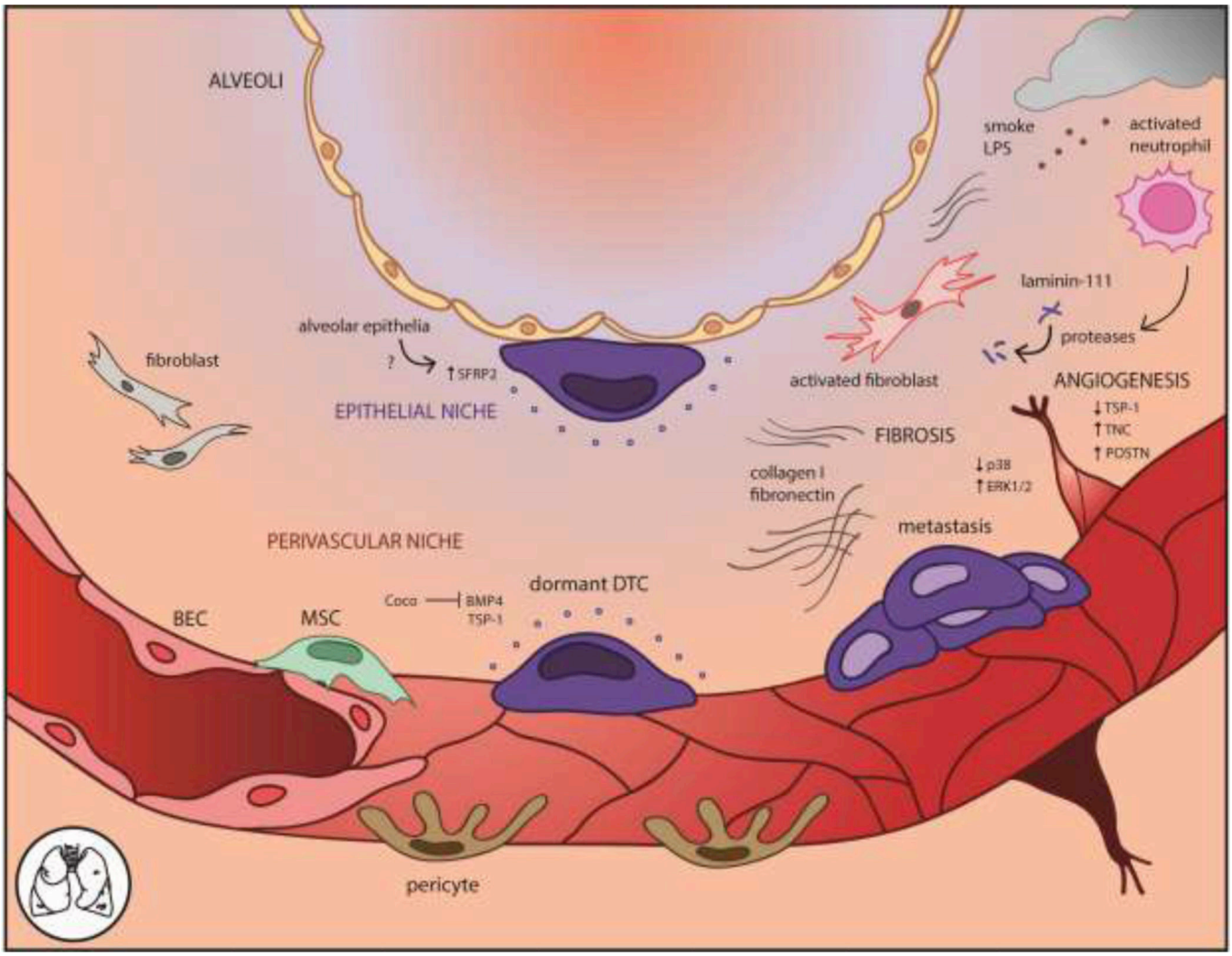

Figure 2. The lung microenvironment for dormant DTCs includes the perivascular and epithelial niches.

In the perivascular niche, deposited basement membrane components like TSP-1 induce cell cycle arrest. The lungs are rich in BMP signaling and BMP4 specifically promotes DTC quiescence, which is antagonized by the BMP inhibitor Coco. The epithelial niche induces SFRP2 in DTCs through an unknown factor. Environmental insults such as tobacco smoke or bacterial LPS elicit inflammation, leading to pro-metastatic angiogenesis, fibrosis, and ECM alterations such as cleavage of laminin-111 by activated neutrophil proteases. These changes are associated with metastatic outgrowth of dormant DTCs.

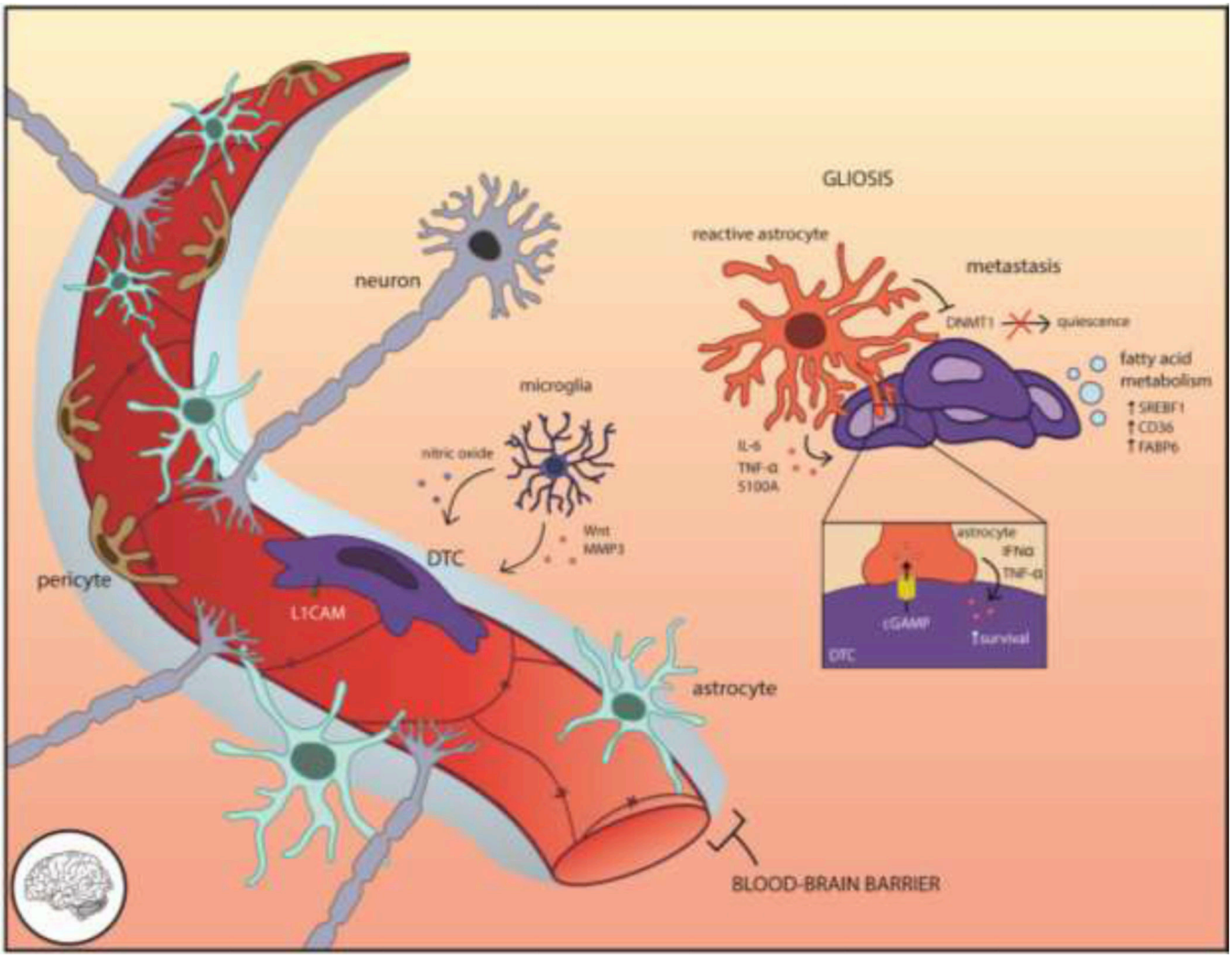

Figure 3. The brain microenvironment requires DTCs to adapt in order to survive.

Access to the brain parenchyma is restricted by the blood-brain barrier (BBB), a tightly reinforced layer including endothelia and concentric layers of supporting pericytes, astrocytes, and neurons. DTCs that manage to extravasate through the BBB rely on L1CAM-mediated spreading on the vasculature to trigger YAP-dependent survival. Astrocytes maintain the integrity of the vasculature and repel invasive cells through Fas ligand but undergo gliosis as an injury response. Reactive astrocytes secrete pro-metastatic cytokines such as IL-6, TNF-α, and S100a and exchange factors directly with DTCs through gap junctions, which elicits paracrine survival signaling (IFNα, TNF-α) back to the DTC. Microglia eliminate tumor cells through nitric oxide but also contribute pro-invasive Wnts and MMP3. The unique lipid composition of the brain necessitates DTCs adapt their fatty acid metabolism through SREBF1, CD36, or FABP6 for successful colonization.

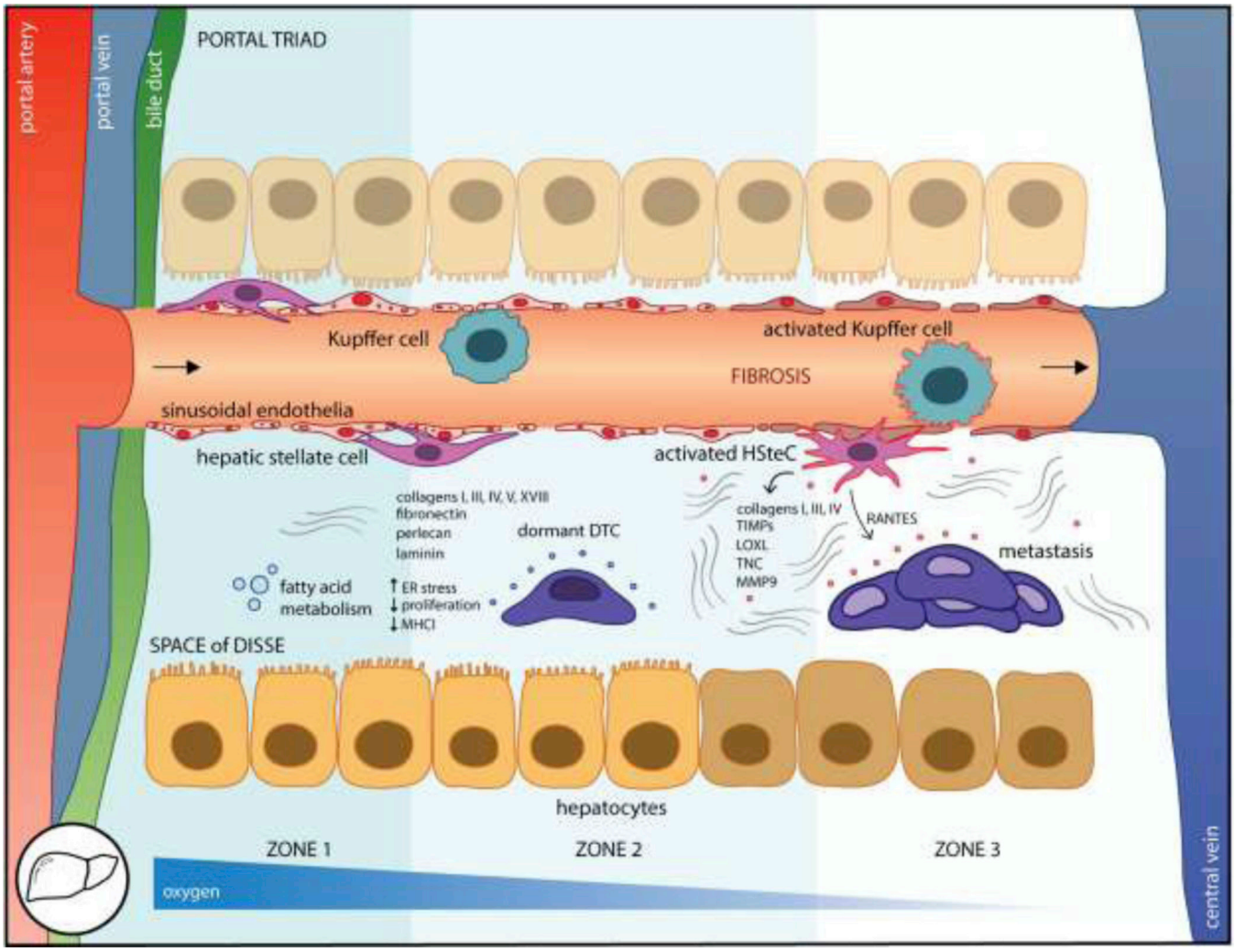

Figure 4. The liver is the most common major metastatic organ.

DTCs traffic from the portal triad vessels to the fenestrated sinusoids and into the subendothelial space of Disse where they encounter stromal cells including liver sinusoidal endothelial cells (LSEC), hepatic stellate cells (HSteC), Kupffer cells, and hepatocytes. Distinct zones occur along the portal triad to central vein axis following oxygen and functional gradients. The loosely organized fibers and proteoglycans basement membrane include collagens, fibronectin, perlecan, and laminin which impose quiescence. Metabolic adaptation slows DTC proliferation, although it also coincides with downregulation of MHCI and immune evasion. However, upon inflammation or injury, the liver become fibrotic which is associated with metastatic outgrowth. Activated HSteCs deposit vast quantities of ECM fibers and other pro-metastatic factors such as RANTES, endothelia undergo capillarization, and hepatocytes lose their microvilli. These changes to the liver microenvironment alter the niche to foster metastasis.

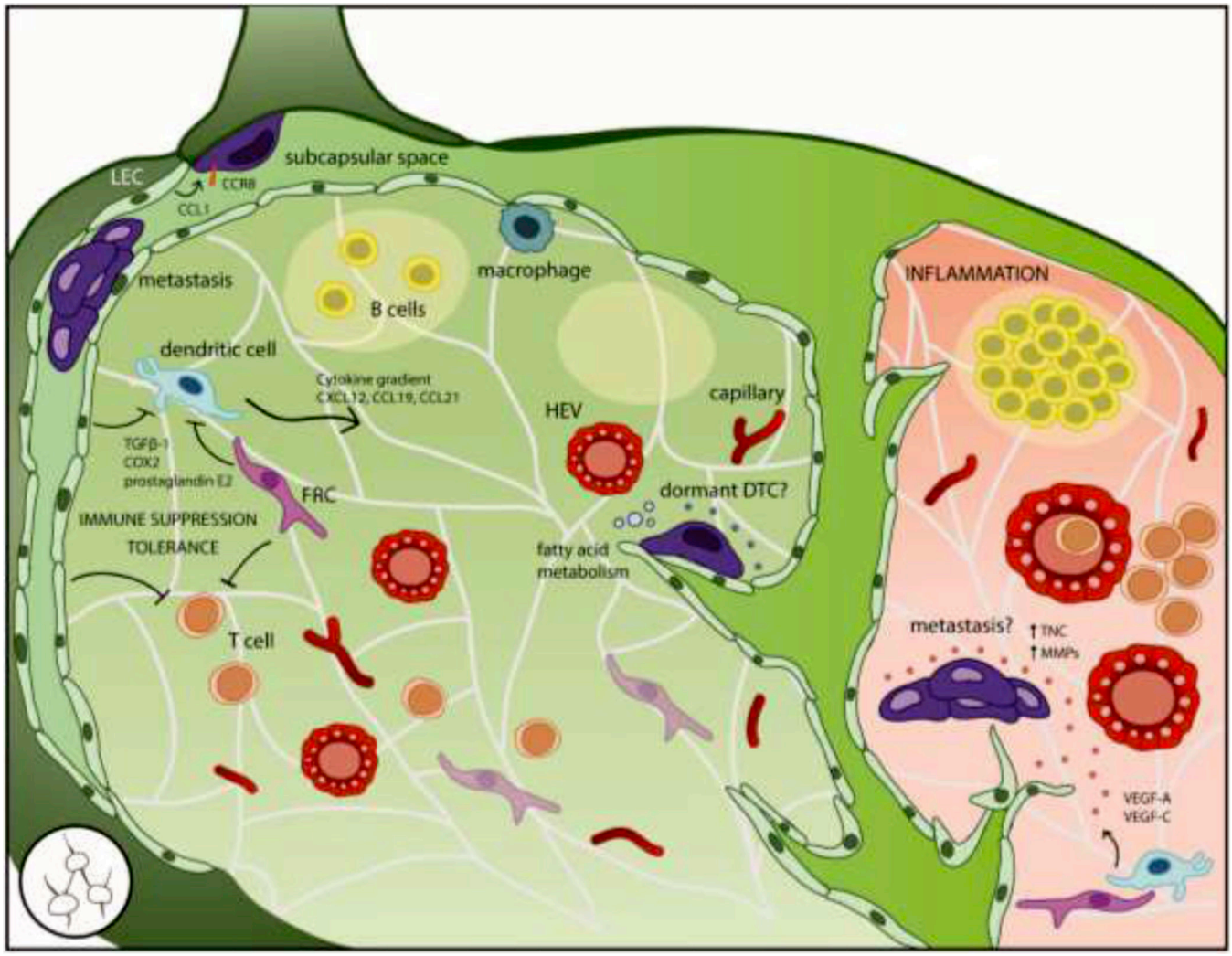

Figure 5. The lymph node (LN) is the most common site of all metastases but its role as a dormancy niche is unknown.

The LN is highly compartmentalized to support its primary function in peripheral immunity. In addition to resident and migrating immune cells, the LN stroma includes lymphatic endothelial cells (LECs) that form the outer capsule and draining sinusoids, blood endothelial cells (BECs) that form capillaries and specialized high endothelial venules (HEVs), and fibroblastic reticular cells (FRCs) that produce an ECM conduit network to facilitate antigen sampling. DTCs frequently access the LN by lymphatic or hematogenous routes, but must adapt to the LN microenvironment through metabolic pathways and co-opting signals that guide immune cells into the LN parenchyma such as CCL1-CCR8 signaling at the afferent lymphatic vessel and cytokine gradients of CXCL12, CCL19, and CCL21; otherwise, they risk remaining confined to the subcapsular space. LN DTCs may benefit from immune suppressive and tolerogenic functions of FRCs and LECs. However, the stromal components that contribute to a dormancy niche have not been formally described. During inflammation, the LN activates into a proliferative environment including

angiogenesis and lymphangiogenesis; these changes to the stroma could conceivably induce outgrowth of dormant DTCs, but must be more fully investigated.