REVISITING SEED AND SOIL: A NEW APPROACH TO TARGET HIBERNATING DORMANT TUMOR CELLS,
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2nd Edition

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Editorial: Revisiting seed and soil: A new approach to target hibernating dormant tumor cells

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Editorial on the Research Topic
Revisiting seed and soil: A new approach to target hibernating dormant tumor cells

Over hundred and thirty years have passed since Stephen Paget proposed his 'Seed and Soil' hypothesis about metastatic spreading (1). Since then, we have gained a better insight into the complexities of this multistep dispersal, but we are yet to fully comprehend the interactions governing the metastatic ecosystems to enable us to clinically prevent disease recurrence and, ultimately, cancer-related deaths.

Despite the high inefficiency of the metastatic process (2), cancer patients often suffer late recurrences following five to thirty years of undetectable disease (3, 4). This clinical observation is due to the presence of disseminated tumour cells (DTCs) that escape early from the primary tumour and spread to distant organs. Once there, DTCs may initially lie in a dormant or hibernating state to later reawaken, resulting in incurable metastatic outgrowths (5, 6). Dormant DTCs persist in a non-proliferative but reversible arrest, and consequently are resistant to conventional therapies directed towards rapidly dividing cancer cells. Moreover, they adapt to the metastatic niche they reside in, evading the immune system and reactivating tumour-initiating abilities when the opportunity arises (7, Weidenfeld and Barkan) (see (7) for concept definitions to avoid terminology misconceptions).

Therefore, metastatic dormancy represents a major clinical problem, as well as a novel window of opportunity to hamper metastatic relapse by interfering with the dormant cancer cell life cycle [key steps to control dormancy in (7)].

In this Research Topic, we revisited Paget's hypothesis focusing on the dormant phase of metastatic progression. This 10-article collection provides an overview on recent advances in the dormancy field, including original research on organ-specific mechanisms driving the reawakening of DTCs; and comprehensive and exhaustive reviews about the microenvironmental regulation of dormancy and reawakening, cutting-edge technologies to study interactions with the metastatic microenvironment and innovative therapeutic strategies to clinically monitor and target this undetectable stage of metastatic disease (Figure 1).

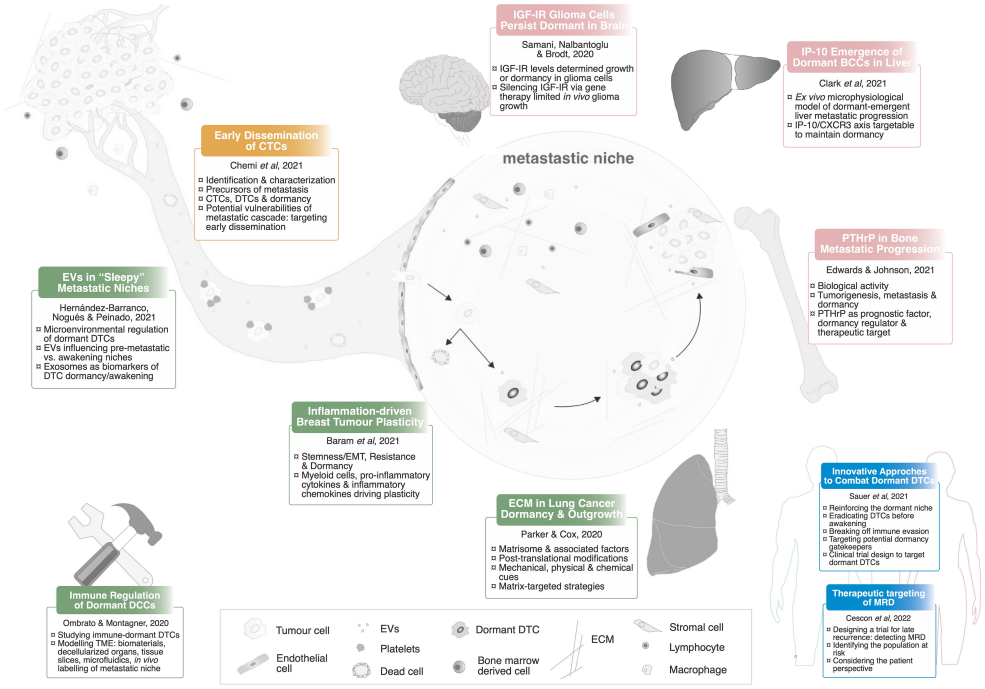

FIGURE 1
Revisiting the Seed and Soil hypothesis. All the articles included in the Research Topic are summarised in this schematic representation. CTCs, Circulating Tumour Cells; EVs, Extracellular Vesicles; DTCs, Disseminated Tumour Cells; EMT, Epithelial-to-Mesenchymal Transition; TME, Tumour Microenvironment; ECM, Extracellular Matrix; MRD, Minimal Residual Disease.

Early dissemination

The journey of tumour cells with metastatic potential begins with their dissemination through the bloodstream and/or the lymphatic system. These traveller precursors of metastases, known as circulating tumour cells (CTCs), are extensively discussed by Chemi et al., who highlight their heterogeneity and suggest their potential utility as non-invasive biomarkers (liquid biopsy) to track minimal residual disease. Although initial spreading follows blood-flow patterns and vascular architecture (8) and few CTCs extravasate into secondary organs (9), Chemi et al. suggest that CTC molecular profiling could predict organotropism to a specific metastatic site.

Organ-specific awakening of dormant DTCs

Most evidence we have gathered over years of research in the field shows intrinsic properties of the seeds in regulating dormancy at specific secondary sites. Molecular players such as TGFβ/BMP, p38/MAPK, NR2F1, uPAR, β1-integrins, IL-1β, among others, have been linked to dormancy or reawakening of metastatic cells in specific organs and cancer types (10–14). However, not all is black and white. In bone, Edwards and Johnson discuss in a comprehensive perspective that molecules such as PTHrP play opposing roles at different stages of disease progression and metastasis. In addition, there are two original research articles that highlight organ-specific molecular mechanisms involved in reawakening. Samani et al. show that downregulation of IGFR-I receptor limited glioma growth, promoting a dormant phenotype; Clark et al. use an *ex vivo* all-human liver microphysiological model to find that IP-10 promotes dormancy exit in the liver metastatic niche.

Microenvironmental regulation of metastatic dormancy

Although Paget's static notion drew attention to the importance of the surroundings (the soil), his hypothesis lacks evolving adaptation that occur in the metastatic ecosystem. The plasticity of the soil, either the tumour microenvironment (TME) or specific metastatic niches, is essential to understand the intricacies of metastatic progression, from primary tumour escape and intravasation, through CTC survival and extravasation at secondary sites, homing and DTCs survival to eventual colonisation.

Seeds and soils are not hermetic compartments, they reciprocally interact with and modulate each other. In fact, the primary tumour can even influence secondary organs to prepare the "congenial soil" or pre-metastatic niche *via* secreted factors and shed extracellular vesicles (EVs), preceding the arrival of the seeds (15). Hernández-Barranco et al. emphasise the need to unravel the communication mechanisms between DTCs and their metastatic niche, suggesting that secreted EVs could mediate this microenvironmental crosstalk to regulate dormancy. The authors also discuss the participation of EVs in the awakening of dormant metastatic cells as well as their potential utility as biomarkers to monitor minimal residual disease, suggesting a step forward for the liquid biopsy field.

Besides interacting with stromal cells, DTCs also display bidirectional communication with another essential structural element of the surroundings, the extracellular matrix (ECM). This three-dimensional network, consisting of macromolecules (such as proteins, proteoglycans, glycoproteins, cytokines and growth factors) modulates its own remodelling during the metastatic process (16–18). In fact, some ECM proteins such as tenascin C, periostin, type-I and type-XII collagen, among others, promote metastatic colonisation in different organs (19–22), whereas recent studies report that other ECM proteins such as thrombospondin-1, fibronectin, laminin-211 and type-III collagen sustain metastatic dormancy (23–26). Here, Parker and Cox exhaustively review the role of the ECM and associated factors in the regulation of tumour dormancy and metastatic outgrowth using the lung extracellular matrix as an example. The authors highlight that developing organ-specific ECM targeting strategies could reduce lung metastatic burden in lung and other solid cancers.

Recent advances in immunotherapy highlighted the role of the immune system in targeting cancer cells and therefore preventing metastasis. Although dormant metastatic cells evade both the innate and adaptive immunity (27) *via* intrinsic downregulation of activating receptors (28, 29), some immune populations such as NK cells contribute to sustaining a dormant phenotype at secondary sites (30). More details about the immunoregulatory control of survival and outgrowth of dormant DTCs can be found in Ombrato and Montagner. Another interesting aspect is the inflammatory microenvironment and its contribution to disease progression and metastasis (27). Baram et al. reviews recent findings on how inflammation (both driven by associated myeloid cells and other factors of the TME) influences tumour cell plasticity, focusing on the regulation of three areas: stemness and EMT, resistance to therapies and dormancy.

Experimental model systems

However, this complex communication occurring within the metastatic ecosystem requires the development of cutting-edge techniques to dissect specific niche interactions and to study dormant DTCs, aiming for the discovery of more specific targets to prevent recurrence. Recent technological advances for studying interactions in the metastatic TME *in vitro* and *in vivo* are discussed by Ombrato and Montagner. The detailed list includes the use of biomaterials, decellularized organs, tissue slices, microfluidics and niche-labelling techniques.

Therapeutic targeting of dormant metastatic cells

Once metastatic relapse occurs, cancer patients have very limited treatment options, and those few available choices usually rely on primary tumour features. Without doubt, metastatic dormancy provides a new window of opportunity to prevent relapse (10, 11, 31). But can we impact the clinical management of metastasis? And, most importantly, can we translate the lessons learnt at the bench into clinical practice? Sauer et al. and Cescon et al. give us clinical perspectives about potential strategies to target dormant DTCs and monitoring of patients with no evidence of disease. Interestingly, Sauer et al. highlight drug repurposing as a therapeutic approach in this context. Furthermore, both articles consider the patient perspective to explore in detail the challenges and shortcomings of current clinical trial design to target this early stage of metastasis.

Nevertheless, some of the questions posed by Paget more than a century ago remain unanswered: "What is it that decides what organs shall suffer in case of disseminated cancer?" (1). Hopefully, we are closer (just few steps away) to unravelling the complexity of the metastatic ecosystems. We invite the reader to enjoy this Research Topic containing current hot topics in the field of dormancy of disseminated tumour cells.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

DB is a consultant for VujaDe Sciences. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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The Role of the ECM in Lung Cancer Dormancy and Outgrowth

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The dissemination of tumor cells to local and distant sites presents a significant challenge in the clinical management of many solid tumors. These cells may remain dormant for months or years before overt metastases are re-awakened. The components of the extracellular matrix, their posttranslational modifications and their associated factors provide mechanical, physical and chemical cues to these disseminated tumor cells. These cues regulate the proliferative and survival capacity of these cells and lay the foundation for their engraftment and colonization. Crosstalk between tumor cells, stromal and immune cells within primary and secondary sites is fundamental to extracellular matrix remodeling that feeds back to regulate tumor cell dormancy and outgrowth. This review will examine the role of the extracellular matrix and its associated factors in establishing a fertile soil from which individual tumor cells and micrometastases establish primary and secondary tumors. We will focus on the role of the lung extracellular matrix in providing the architectural support for local metastases in lung cancer, and distant metastases in many solid tumors. This review will define how the matrix and matrix associated components are collectively regulated by lung epithelial cells, fibroblasts and resident immune cells to orchestrate tumor dormancy and outgrowth in the lung. Recent advances in targeting these lung-resident tumor cell subpopulations to prevent metastatic disease will be discussed. The development of novel matrix-targeted strategies have the potential to significantly reduce the burden of metastatic disease in lung and other solid tumors and significantly improve patient outcome in these diseases.

Keywords: extracellular matrix, lung cancer, dormancy, metastasis, matrix-targeting

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Abbreviations: ADAMTS, A Disintegrin and Metalloproteinase with Thrombospondin motifs; Akt, v-akt murine Thymoma Viral Oncogene Homolog; AMPK, AMP-activated protein kinas; AXL, AXL Receptor Tyrosine Kinase; BH3, BCL2 Homology Region 3; BMP, bone morphogenetic protein; CD44, cluster of differentiation 44; CDC42, cell division control protein 44 homolog; COL2A1, collagen type II alpha chain; COL5A2, collagen type V alpha chain 2; COPD, Chronic Obstructive Pulmonary Disorder; CXCR1, C-X-C motif chemokine receptor 1; DKK1, Dickopf WNT signaling pathway inhibitor 1; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FACIT, fibril associated collagens with interrupted triple helices; FAK, focal adhesion kinase; FGF, fibroblast growth factor; FGF2, fibroblast growth factor 2; GAS6, growth arrest -specific protein 6; HAPLN1, Hyaluronan and Proteoglycan link protein 1; HB-EGF, Heparin-binding EGF-like growth factor; HGF, hepatocyte growth factor; HIF1a, hypoxia inducible factor 1; HOXB9, homeobox protein B9; IL6, interleukin 6; IPF, idiopathic pulmonary fibrosis; JAK2, Janus Kinase 2; JNK, c-Jun N-terminal kinase; LEF1, lymphoid enhancer-binding factor 1; LKB1/STK11, liver kinase B1 or serine/threonine kinase 11; LOX, lysyl oxidase; LOXL2, lysyl oxidase like-2; MEK, mitogen activated protein kinase kinase; MIF, macrophage migration inhibitory factor; MLC, myosin light chain; MLCK, myosin light chain kinase; MMP9, matrix metalloproteinase 9; MSI1, RNA-binding protein Musashi homolog 1; mTOR, mammalian target of rapamycin; NK, natural killer cell; NSCLC, non-small cell lung cancer; PDGF, platelet derived growth factor; PET, positron emission tracer; PGP, proline-glycine-proline tripeptide; PI3K, phosphatidylinositol 3-kinase; Raf, rapidly accelerated fibrosarcoma; ROCK, rho-associated coiled -coil kinases; SOX2, SY-box transcription factor 2; STAT5, signal transducer and activator of transcription 5; TAZ, tafazzin; TGFb, transforming growth factor beta; TLR, toll like receptor; TNFa, tumor necrosis factor alpha; uPA, Urokinase-type plasminogen activator; VCAM-1, vascular cell adhesion protein 1; VEGF, vascular endothelial growth factor.

LUNG CANCER DYNAMICS AND DORMANCY

Lung cancer remains the leading cause of cancer death worldwide (1). Non-small cell lung cancer (NSCLC), which consists of adenocarcinoma, squamous cell carcinoma and large cell carcinoma, accounts for more than 80% of all lung cancer cases (2). Adenocarcinoma and squamous carcinoma are the predominant NSCLC subtypes. Large cell carcinoma represents less than 5% of NSCLC cases and is diagnosed when the tumor cannot be identified by conventional adenocarcinoma and squamous markers. Large cell NSCLC is increasingly viewed as an undifferentiated form of squamous carcinoma and adenocarcinoma rather than a distinct histological subtype, as more sophisticated molecular characterization of these tumors identifies features of the adenocarcinoma or squamous carcinoma subtypes within these tumors. Adenocarcinoma and squamous carcinoma have distinct anatomical growth patterns, with adenocarcinoma and squamous carcinoma developing in the distal and central lung, respectively. NSCLC subtypes are believed to arise from different cells of origin in a context-dependent manner (3) although the etiology of non-small cell lung cancer subtypes remains to be precisely defined.

Effective management of NSCLC is hampered by the late presentation of the disease, when metastatic foci are typically already established. The major sites of non-small cell lung cancer metastasis are the brain (12–47%), bone (16–39%), liver (7–22%), intrapulmonary (to contralateral or ipsilateral lobes, 11–26%), pleura (10–13%), thoracic lymph node (29%) and adrenal gland (6–15%) (4–7). The specific organ tropism of lung cancer cells for these secondary sites partly depends on the histology and genomic profile of the primary tumor (8, 9). While the metastatic profile of adenocarcinoma and squamous carcinoma are similar, adenocarcinoma has a higher incidence of bone metastases and intrapulmonary metastases than squamous carcinoma (5, 10), suggesting that adenocarcinoma and squamous carcinoma cells, either intrinsically or through their interaction with their tumor microenvironment, are differentially programmed with regards to metastatic propensity and organ tropism. This review will unravel the current understanding of how the extracellular matrix of the primary lung tumor and secondary sites regulate the formation of metastatic foci in non-small cell lung cancer.

Aided by improved detection modalities (11, 12), lung cancer is increasingly being diagnosed during the early stages of progression, where curative-intent surgical resection is the front line therapy and is associated with significantly greater 5 year survival than disseminated disease. However, disease recurrence is prevalent even among early stage (stage I and II) NSCLC patients, with 30–60% of early stage patients with margin-negative resected lung cancer developing local or distant disease recurrence (13, 14). NSCLC has a relatively high rate of synchronous (25%) and metachronous (2% per year) tumors. These tumors may develop from multiple independent tumor initiating events or through contralateral or ipsilateral metastasis of a primary lung tumor, making it difficult to determine the true rate of intrapulmonary metastasis in NSCLC (15, 16). These etiologies are distinct processes, with the former occurring when multiple tumors are initiated independently in the lung but emerge metachronously due to differential reawakening of the dormant transformed cells *in situ*. Conversely, the latter occurs *via* a conventional metastatic process whereby cells disseminate away from the primary tumor to colonize a niche within the lung that is anatomically distinct from the primary tumor site.

Emerging evidence from DNA sequencing studies mapping the clonal evolution of lung tumors is providing unprecedented insight into the dynamics of lung tumor outgrowth, as well as distinguishing between tumors arising from intrapulmonary metastases or from independent transformation events. Correlations between multiregional tumor sequencing and smoking-associated behavior suggest that driver gene mutations occur several decades prior to cancer diagnosis (17) and therefore that primary and secondary NSCLC tumors are likely to undergo some period of dormancy before becoming re-awakened. Furthermore, intrapulmonary metastases are associated with a longer latency than distant metastases, commonly re-emerging more than 5 years following surgery (13). In addition to genetic changes, widespread and dispersed changes in the structure and composition of the lung ECM as well as the transcriptional profile of normal bronchial epithelia in smokers and lung cancer patients are thought to represent a field of cancerization that promotes tumor initiation and regulates the dissemination of lung tumor cells from the primary site (18–22). Similarly, the severe extracellular matrix remodeling in chronic lung diseases such as chronic obstructive pulmonary disorder (COPD) and idiopathic pulmonary fibrosis (IPF), which are associated with an increased risk of lung cancer development, may also contribute to this field effect (23, 24). Although the mechanisms underlying these clinical associations remain unclear, these associations support the notion that the extracellular matrix is an important regulator of NSCLC etiology.

The mechanisms that drive the dormancy and reawakening of lung cancer cells both within the lung and in other secondary organs remain to be precisely defined, however, there is a clear tissue tropism to the induction, maintenance and re-awakening of tumor cell dormancy that occurs in a cancer type-dependent manner (25, 26). The extracellular matrix is well recognized as a regulator of cellular proliferation and differentiation. Studies in other cancers have revealed mechanisms by which the matrix regulates this dormancy and the outgrowth of metastases, and these molecular alterations are also seen in lung cancer. As such, valuable insights into the dormancy and metastatic behavior of primary lung tumors come from studies of the metastatic colonization of the lung by non-pulmonary cancer cells, as well as studies of both primary and metastatic lung cancer. The importance of the extracellular matrix in regulating dormancy and re-activation is emerging as an important area of research, and a resource from which novel therapies targeting metastasis are being developed. This review addresses our current understanding of the role of the extracellular matrix in regulating the dormancy and emergence of both primary and secondary lung tumors.

THE ROLE OF THE ECM IN PRIMARY AND SECONDARY DORMANCY DYNAMICS

The accredited model of tumor dynamics is that disseminated cancer cells interact with both local and distal stromal and immunological cells through systemic and local signaling to establish niches that support the engraftment and survival of cancer cells. The seed and soil hypothesis states that for successful metastasis to occur, disseminated tumor cells must be compatible with the secondary niches that they go on to colonize. Disseminated tumor cells may be maintained in these metastatic niches in a dormant or quiescent state, and cells that are compatible with their new environment may later re-enter the cell cycle and start proliferating to form metastatic foci in response to intrinsic and microenvironmental cues (27). The extracellular matrix and associated factors engage with cell surface receptors to regulate intracellular signaling programs that control this exit and re-entry to the cell cycle in a context- and cell type- dependent manner. An emerging understanding of how these processes are co-ordinately regulated by the tumor microenvironment is challenging claims that this quiescence occurs stochastically (28).

The extracellular matrix of the lung incorporates a diverse group of core matrisomal proteins that form the structural basis of the tissue, together with the enzymes responsible for remodeling and processing these molecules, as well as the soluble factors that are associated with this matrix (29) (**Figure 1**). Together, these matrisomal proteins function as a dynamic network of structural and signaling effectors that undergo constant remodeling. The function of the central airways and distal parenchyma are profoundly shaped by regional differences in the extracellular matrix composition and architecture of these compartments (**Figure 1A**). Associations between the ECM composition of NSCLC tumors and the risk of disease recurrence (30), points to a role for the extracellular matrix in regulating cancer cell dissemination, dormancy and outgrowth in intrapulmonary and distant metastases.

While mechanisms of dormancy appear strongly cell-type and context dependent, these pathways largely converge on mitogenic and stress response signaling pathways. In particular, the TGFβ/BMP, FAK, Src, uPA, EGFR, and integrin signaling pathways are integrated with LKB1/AMPK, PI3K and metabolic signaling to modulate ERK/p38, JNK, cyclin and downstream cell cycle regulators to control the entry and exit of cells from the cell cycle (27, 31, 32) (**Figure 1D**). For example, integrin receptor and uPAR activation converge on the ERK/p38 pathway where low ERK1/2 activity coupled with high p38 activity drives a dormant phenotype (33) through transcriptional regulation that is associated with poor progression-free survival in numerous cancers (34) (**Figure 1E**). Crosstalk between integrin signaling and the EGFR and uPAR pathways, which intersect with the downstream effectors of integrin receptors, including FAK, also regulate dormancy by these same p38 and p27-dependent pathways, as well as through Akt signaling cyclin D1 activation (35, 36). In addition, stem cell programs characterized by pro-survival, quiescent signaling also support dormancy and metastasis. For example, cues from the extracellular matrix interact with Wnt and Notch signaling to mediate these quiescent phenotypes (**Figures 1D,E**). How these pathways operate specifically in lung cancer remains less clear than in other well-studied cancer types such as breast cancer (37). However, concordant transcriptional signatures uncovered in breast metastases within the lung and models of aggressive metastatic lung cancer (38) suggest that common mechanisms involving the ECM may operate across cancer types. Understanding the effect of the extracellular matrix in orchestrating these intersecting dormancy signaling pathways lays the foundation for developing therapeutic approaches to improve lung cancer treatment.

The Core Matrisome

The extracellular matrix composition of the central lung is dominated by fibrillar collagens (primarily collagen types I and II), while the interstitial ECM of the alveoli in the distal lung is a relaxed network of mainly type I and II collagens and elastin (**Figure 1A**). Compared with normal lung tissue, primary lung tumors display significant changes in the core matrisomal proteins that maintain the structural and mechanical features of the tissue. Due to their different anatomical locations, adenocarcinoma and squamous cancer cells are exposed to different extracellular matrix environments (**Figure 1A**), which likely shape the evolution of these tumor types and thus contribute to differences in etiology. While direct comparisons of the ECM landscape of adenocarcinoma and squamous carcinoma with respect to peripheral and central ECM composition in healthy lung have not yet been performed, consideration of the ECM composition in different lung anatomical compartments will be important in identifying shared ECM remodeling programs that generally contribute to lung tumorigenesis across multiple subtypes. Even within these broad histological subtypes, the extracellular matrix landscape differs between distinct molecular subclasses of adenocarcinoma and squamous carcinomas (39). Whilst oncogenic driver mutations are known to contribute to some of this heterogeneity in both the primary and secondary NSCLC tumors, it is now clear that our understanding should include extracellular matrix components that also modulate tumor heterogeneity. Precisely how these anatomical differences in the lung affect non-small cell lung cancer dormancy dynamics, and indeed whether they play a role in the preferential colonization of the lung by non-pulmonary tumor cells, remains unclear. Emerging evidence does, however, point strongly to roles for the core matrisome composition and architecture in regulating the dormancy and outgrowth of cancer cells within the lung as well as at other common NSCLC secondary sites (**Table 1**).

Collagens

Collagens are a significant component of the lung ECM and altered collagen composition and structure are known to regulate cancer cell dormancy. Primary lung tumors of both the adenocarcinoma and squamous carcinoma subtypes have increased fibrillar collagen deposition and an altered collagen architecture that is consistent with a fibrotic response (40)

(**Figure 1B**). In breast cancer models, increased fibrillar collagen activates β1- and β4- integrin signaling to promote the colonization and proliferation of metastatic foci in the lungs (41) as well as the re-awakening of disseminated dormant tumors cells (42), and as such, similar mechanisms are expected to operate in primary NSCLC tumors. Increased expression of type IV collagen, and decreased expression of collagen III and collagen XVIII are associated with NSCLC liver metastases, where they activate pro-survival α2β1-integrin signaling to sustain NSCLC cancer cells within the liver (43), although the mechanisms driving these changes are unknown. In primary lung adenocarcinoma, fibrosis seen in mediastinal lymph node metastases resembles that of the primary lung adenocarcinoma (40), suggesting that either intrinsic features of the primary cancer cells promote a fibrotic environment at secondary sites through crosstalk with stromal cells, or that aberrant fibrosis at secondary sites may promote overt outgrowth of the disseminated cells that drain there.

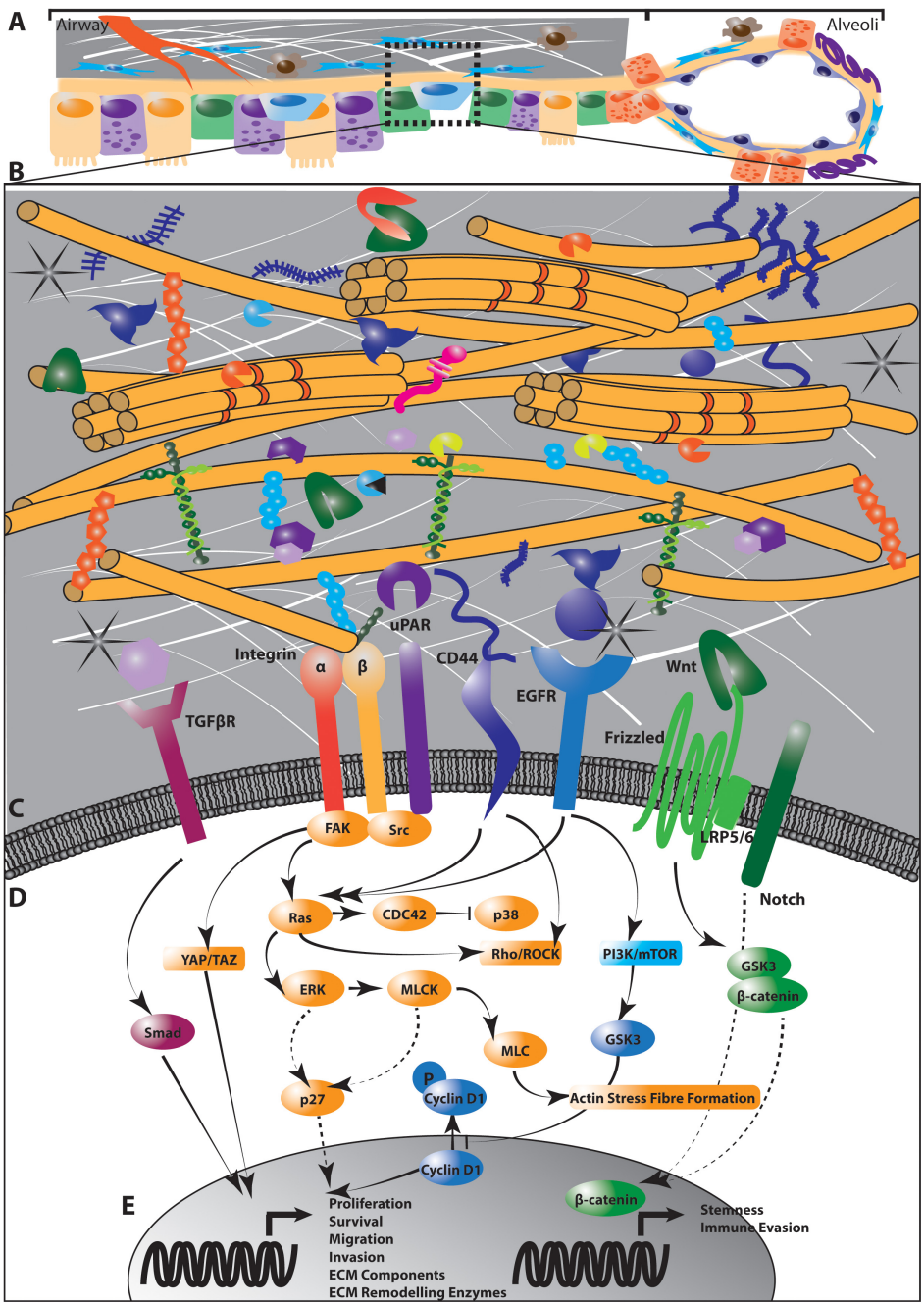

FIGURE 1 | Continued

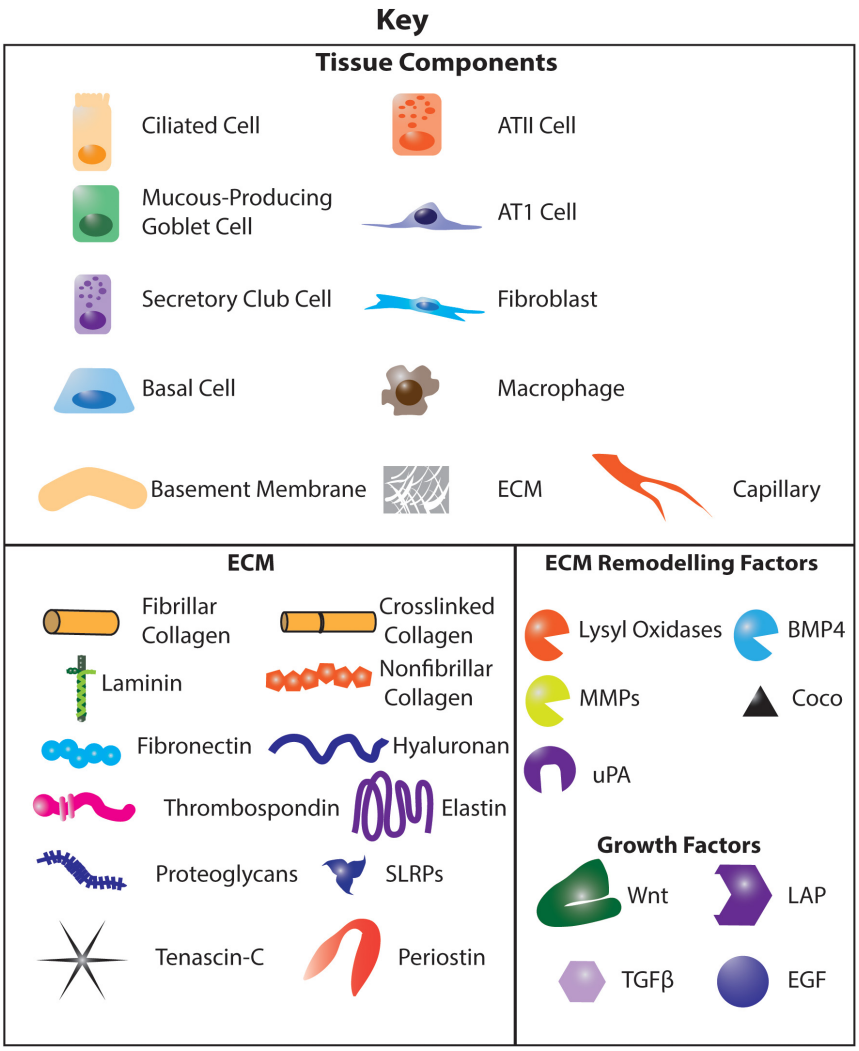

FIGURE 1 | The interaction of cancer cells with the extracellular matrix (ECM) in the lung regulates diverse intracellular signaling pathways to regulate cancer cell dormancy, proliferation and metastases in NSCLC. Multiple cell types within the lung generate and remodel the extracellular matrix **(A)**. The composition of the ECM differs from the central airways and peripheral parenchyma where alveoli enable gas exchange, and is highly dysregulated in NSCLC **(A)**. This ECM is composed of matrisomal proteins, factors that associate with these proteins as well as enzymes involved in remodeling this matrix **(B)**. The posttranslational modification of ECM proteins and their interaction with each other ECM components determines the biomechanics of the tissue. ECM remodeling also releases growth factors and reveals cryptic sites within ECM components that are recognized by cells. Receptors on the cell membrane directly interact with specific ECM components and secreted factors **(C)** to activate diverse intracellular signaling programs that regulate the proliferative and metastatic characteristics of cancer cells **(D)**. These signaling programs drive the transcription of cell cycle regulators, stem cell markers and genes involved in EMT, migration, invasion and immune activation to regulate tumor dynamics **(E)**. In addition, these signaling programs activate the expression of ECM genes and ECM remodeling enzymes to drive further evolution of the tumor microenvironment **(E)**. Dotted lines indicate indirect interactions. ATII, Type II pneumocyte; ATI, Type I pneumocyte.

Whilst changes in the composition and architecture are the major collagen alterations in lung tumors, mutations in collagen type V alpha 2 chain (COL5A2) and collagen type II alpha I chain (COL2A1) genes have also been detected in subclonal secondary lung tumors following genomic doubling events in both adenocarcinoma and squamous carcinoma (44). While somatic mutations in extracellular matrix components are considered rare, and of lesser influence on the tumor extracellular matrix landscape than dynamic compositional and architectural changes, the effect of these collagen mutations on the extracellular matrix architecture and function remain to be investigated.

The collagen composition of tissues is partly determined by the activity of proteases that process nascent collagen molecules for assembly into the 3D environment. Bone Morphogenetic Protein 1 (BMP1) and members of the A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) family, which are differentially expressed in NSCLC tumors, are required to cleave the N- and C-termini of the pro-collagen peptide to enable collagen fibrillogenesis and deposition (45). It is not clear yet how altered expression of these proteases within the primary tumor might influence the increased deposition of fibrillar collagens in NSCLC. In addition to changes in

TABLE 1 | Summary of functional studies identifying a direct role for ECM components in Dormancy and outgrowth in solid cancers affecting the lung.

ECM category	ECM component	Role	Experimental system
Collagens	Collagens	Increased fibrillar collagen levels promotes proliferation, metastasis and outgrowth	*In vivo* models and human biospecimens (40)
		Increased type IV collagen, decreased type III and type XVIII collagens promote liver metastasis and outgrowth	*In vivo* models and human biospecimens (43)
Glycoproteins	Laminin	Laminin-cleavage promotes dormant re-awakening	Metastatic breast cancer model (61, 62)
	Fibronectin	Increased fibronectin promotes lung colonization and outgrowth, NSCLC proliferation and invasion	Metastatic lung cancer mouse model (66) Metastatic breast cancer mouse model (68) Biospecimens of metastatic human breast cancer (67) *In vitro* NSCLC models (70, 71)
	Tenascin-C	Promotes adenocarcinoma metastasis	*In vivo* and human biospecimens (40)
		Promotes lung colonization	*In vivo* breast cancer model(80) *In vivo* breast cancer model (84)
	Periostin	High expression is associated with poor prognosis	Human biospecimens (85–88)
		High expression supports lung colonization and outgrowth	*In vivo* breast cancer model (85)
	Thrombospondin	High expression of thrombospondin-2 supports lung colonization and metastasis of primary NSCLC	*in vivo* breast cancer model and human biospecimens (93) human NSCLC biospecimens (95)
		High thrombospondin-1 expression inhibits tumorigenesis	*in vitro* and *in vitro* models (94) *in vivo* NSCLC model (98)
	Osteopontin	High expression is associated with poor survival	Human NSCLC biospecimens (102, 103)
		High expression promotes invasion	*In vitro* models (104, 105)
Proteoglycans and Glycosaminoglycans	Versican	High expression is associated with poor survival	Human NSCLC biospecimens (111)
	Hyaluronan	High expression of hyaluronan or CD44 promotes tumor recurrence	Human NSCLC biospecimens (111, 117)
		High expression promotes tumor proliferation and outgrowth	*in vitro* models, *in vivo* models and human biospecimens (39, 115)

the collagen composition, the fibrillar collagen architecture is altered in NSCLC, becoming more disordered and fragmented at the submicron scale but more aligned at the macro scale compared with normal lung tissue (46). The collagen architecture is regulated at these different length scales by collagen molecules binding to fibronectin, small leucine-rich proteoglycans and Fibril Associated Collagens with Interrupted Triple Helices (FACIT collagens) to nucleate the collagen network (47) as well as the activity of remodeling enzymes (see ECM Remodeling Factors) and cellular traction forces generated by resident cells. However, the mechanisms governing these broad architectural changes in the fibrillar collagen network in lung cancer remain unclear. Aligned collagen fibers seen in higher stage lung tumors would be expected to generate anisotropic biomechanics that have been shown to provide contact guidance cues to regulate cell shape and promote migration in breast cancer models (48). However, how the discrete collagen architecture in the central airways and distal lung contribute to the metastatic behavior of lung cancer cells remains to be investigated.

Collagens, as well as laminin, fibronectin, thrombospondin, osteopontin and other core matrisomal proteins mediate their effects on dormancy by acting as ligands for integrin receptors to activate intracellular proliferation pathways. Integrin receptors are heterodimers of α and β chains that bind to a variety of extracellular matrix molecules, and are the main cellular receptor for collagens. Integrin receptors are capable of bidirectional signaling across the plasma membrane. Intracellular signals regulate the conformation and ligand affinity of the extracellular domain while also mediating intracellular cytoskeletal signaling (35). For example, activation of β1-integrin has been shown to lead to activation of Src and subsequently FAK and ERK signaling via Ras to regulate dormancy (**Figure 1D**). The subsequent activation of MLCK in this signaling cascade also regulates actin dynamics and stress fiber formation (42), driving the translocation of p27 into the cytoplasm to initiate cell proliferation and re-entry of dormant cells into the cell cycle (49). Simultaneously, integrin receptor engagement activates CDC42, which inhibits p38 expression, thus resulting in a high ERK/low p38 profile that supports continued cell proliferation. Conversely, loss of receptor engagement drives a low ERK/high p38 profile that favors tumor cell dormancy, while negatively feeding back onto ERK activation to inhibit cell proliferation (50). Integrin signaling also has significant crosstalk with growth factor signaling. For example, EGFR signaling and activated integrin receptors can induce ligand-independent activation of EGFR signaling (51) (**Figure 1D**). Whether activating EGFR mutations, found in 15% of adenocarcinoma patients (52) directly perturb integrin signaling responses in NSCLC remains to be seen.

This extensive crosstalk between signaling pathways downstream of integrin receptors generates a network of diverse intracellular signaling pathways that are finely tuned to respond to heterogeneity within the extracellular matrix environment. Specific pairs of α- and β-integrin receptors have different affinities for extracellular matrix components, and enact different downstream signaling events. This creates a signaling program that can be fine tuned to interpret and respond to the subtle shifts in the extracellular environment (53). The collagen-binding integrin subunits β1 and α3 (which also bind

fibronectin, laminin, and thrombospondin), together with the α5- (binds fibronectin), β3- (binds fibronectin, von-willebrand factor, and thrombospondin) and β4- (binds laminin) integrin receptors have been shown to be upregulated or functionally activated by their respective ligands within the lung niche to promote the proliferation and metastatic potential of cancer cells (42, 54–59). In this way, the collagen composition and presence of other integrin ligands in the lung can act as a switch between the dormant and proliferative state (49).

Glycoproteins

Glycoproteins are proteins that are covalently modified with carbohydrate groups. They have diverse functions within the primary tumor, pre-metastatic and metastatic niches including the regulation of dormancy and outgrowth. Below we will discuss the role of some of the most well studied glycoproteins involved in primary and secondary lung tumors.

Laminin

Laminin is a heterotrimeric glycoprotein that acts as an important component of the basement membrane in the lung as well as in other tissues (29). Basement membrane ECM components such as laminin have been shown to play an important role in regulating the cellular proliferative response in both health and disease (**Figure 1A**). Importantly, they are widely understood to promote tumor cell dormancy in non-pulmonary tumors (60).

The laminin-111 isoform in the lung basement membrane is known to regulate the dormancy of breast cancer cells colonizing the lung (61). Albrengues and colleagues found that laminin-111 digestion by neutrophil-derived matrix metalloproteinase-9 (MMP9) and elastase reveals cryptic sites that in turn activate β1-integrin signaling to re-awaken dormant breast cancer cells in the lung (61). This builds upon observations that MMP9 digestion of laminin-111 drives a loss of cell polarity and tumor growth in primary breast cancer models (62). Furthermore, oncogene-driven hyperactive Raf/MEK/ERK signaling induces MMP9 expression in breast cancer cells (62), suggesting that oncogenic features of the cancer cells may accelerate ECM remodeling-dependent mechanisms of reawakening. It is not known if these mechanisms also operate in primary lung tumors or intrapulmonary metastases derived from lung tumors, however, further investigations are warranted since neutrophil infiltration and Raf/MEK/ERK hyperactivation are both features of NSCLC tumors (63, 64).

Fibronectin

The glycoprotein fibronectin directly interacts with multiple extracellular matrix proteins as well as integrin receptors α3, α5, αV, α8, β1, and β3, which together regulate the structure of fibronectin fibrils, cell-ECM engagement and activate pro-proliferative intracellular signaling programs. Under cellular contraction, tensile forces applied to fibronectin reveal cryptic sites that bind to other fibronectin fibers, inducing fibronectin fibril formation that further stretches the fibers (65). These stretched fibronectin fibers act as binding sites for collagen fibers to regulate the fibrillar collagen network architecture. Fibronectin is widely implicated in regulating tumor cell dormancy in breast cancer and melanoma metastasis to the lung by regulating the architecture of surrounding ECM proteins, as well as by direct engagement of integrin receptors (66, 67). Growing evidence suggests that fibronectin may also play a similar role in primary lung cancer progression as well as intrapulmonary metastases.

Fibronectin expression is increased in primary lung tumor sites and at pre-metastatic sites of breast cancer metastasis to the lung (40, 66, 67). In breast cancer models, fibronectin is expressed by fibroblasts in the pre-metastatic niche due to the recruitment and activation of hematopoietic progenitors recruited to the lungs (66, 68). β1-integrin receptors on colonizing cancer cells are then activated upon fibronectin binding. This in turn activates MLCK and actin stress fiber formation that switches breast cancer cells from dormancy to proliferation (49). In primary NSCLC tumors, fibronectin-mediated β1-integrin activation also results in PI3K/Akt and FAK activation to drive pro-proliferative and pro-invasive signaling (69–71). To date, it is not yet clear if the arrangement of fibronectin fibers is significantly altered in NSCLC to regulate focal adhesion formation and FAK activation in a similar manner (72).

In addition to its direct stimulation of integrin signaling, fibronectin can indirectly influence growth factor signaling and cell proliferation by interacting with Bone Morphogenetic Protein-1 (BMP-1), Hepatocyte Growth Factor (HGF), Fibroblast Growth Factor 2 (FGF-2), Platelet-derived Growth Factor (PDGF) and latent TGFβ, although this is yet to be demonstrated specifically in NSCLC tumors (73). It is by these manifold mechanisms affecting cellular proliferation and migration that fibronectin is assumed to regulate lung tumor progression and metastasis. However, the role of this ECM protein in regulating gap junction formation in type II pneumocytes in healthy lung tissue (74, 75) suggests that fibronectin may also influence lung adenocarcinoma tumorigenesis by contributing to altered epithelial structure, cell polarity and potentially self renewal programs in this histological subtype.

Tenascin-C

Tenascin-C is a large multi-domain glycoprotein that undergoes extensive post-transcriptional and post-translational regulation. It is highly expressed in lung development during branching morphogenesis and alveolarization. However, in adult lungs it is only transiently expressed in response to injury (76). Loss of tenascin-C during lung development alters TGFβ signaling, reduces fibroblast to myofibroblast transformation and increases collagen deposition in small airways (76). Conversely, in response to injury, Tenascin-C stimulates TGFβ responsiveness to promote collagen deposition and fibrosis (77). Tenascin-C, like its binding partner fibronectin, is significantly upregulated in fibrotic lungs and in lung adenocarcinoma (40) raising the notion of a potentially overlapping mechanistic contribution to these disease processes. Tenascin-C transcription is also repressed by the canonical lung adenocarcinoma transcription factor Nkx2-1 (40). As adenocarcinoma progresses, Nkx2-1 expression decreases, releasing the suppression of Tenascin-C expression, which is thought to feed into accelerating tumor progression and metastasis (40).

Tenascin-C is also a ligand for β1-containing integrin receptors and is known to activate pro-proliferative integrin

signaling. Together with its close association and interactions with fibronectin, its engagement with integrin receptors is a major mechanism by which this glycoprotein regulates tumor dynamics. Tenascin-C also contains EGF-like domains which can potentially directly activate EGFR signaling (78). While evidence that it directly activates EGFR signaling in NSCLC tumors is currently lacking, it remains possible that Tenascin-C may mediate some of its effects through this mechanism in EGFR wild type tumors. Tenascin-C also binds a number of pleiotropic growth factors, including TGFβ, PDGF, FGF and insulin-like growth factor (IGF) family members, and therefore likely plays an important role in regulating growth factor availability and proliferative signaling within the lung environment (79). Importantly, the role of tenascin-C as a regulator of TGFβ signaling appears to be central to its effects on dormancy in breast cancer cells colonizing the lungs and parallels may be drawn to primary lung tumors or intrapulmonary metastases. Following the dissemination of single breast cancer cells to the lungs, TGFβ signaling is downregulated in dormant cells, switching them into a pro-proliferative phenotype that establishes macrometastatic foci (80). This switch from dormancy to proliferation is determined by TGFβ availability, which is tightly regulated. TGFβ is sequestered to the matrix and remains unavailable by its complexation with the latency associated peptide, which is itself bound to integrin receptors, and latent TGFβ binding proteins, which are tethered to fibronectin, fibrillins, fibulins, proteoglycans and other fibrillar ECM components (73, 81, 82) (**Figure 1B**). This complex holds TGFβ in an inactive state, while its release from these complexes frees TGFβ to bind TGFβ receptors and activate pro-dormancy signaling. By interacting with TGFβ (79), tenascin-C sequesters TGFβ in an inactive state to prevent the induction of pro-dormancy cellular reprogramming and thereby enable proliferative signaling (83) (**Figure 1E**), although it is not clear if this is the central mechanism by which this ECM component contributes to aggressive NSCLC.

High expression of Tenascin-C in breast cancer is associated with increased risk of lung metastases, suggesting that it affects more than proliferative signaling in cancer cells. Here Tenascin-C acts in an autocrine manner on disseminated cancer cells to support their self-renewal, survival and lung colonization characteristics. Tenascin-C-mediated breast cancer cell reprogramming inhibits JAK2-STAT5 signaling to enhance MSI1 expression and drive pro-metastatic NOTCH signaling (84) (**Figures 1C,D**). Whether these interactions occur in subpopulations of cancer cells or equivalently across all cancer cells remains to be determined. As larger metastatic foci form in the lungs, Tenascin-C is increasingly expressed by activated fibroblasts in the metastatic niche (84). How this switch from cancer to fibroblast-mediated expression occurs, and whether these same mechanisms occur in primary and intrapulmonary secondary lung tumors remains unclear. Similar mechanisms observed in bone-tropic breast cancer metastases (84) suggests that Tenascin-C also supports metastatic outgrowth in bone metastases and may contribute to the metastasis of lung cancer cells to the bone microenvironment. Taken together, Tenascin-C appears to play a role in metastatic colonization at multiple sites, both those naturally rich in Tenascin-C and also in tissues that are activated to upregulate Tenascin-C in response to the presence of cancer cells. Further studies are warranted to dissect the mechanisms by which Tenascin-C affects the progression and dissemination of lung tumor cells, with particular attention to where these mechanisms converge with and diverge from its more established role in breast cancer metastasis.

Periostin

Periostin is a secreted glycoprotein that has also been shown to induce awakening of dormant cancer cells within the lung. High expression of periostin, as well as the aberrant expression of alternatively spliced isoforms of this gene, are also associated with poor prognosis in primary lung cancer (85–88). In metastatic breast cancer models, TGFβ2 and TGFβ3 expressed by disseminated tumor cells induce periostin expression in lung fibroblasts and endothelial tip cells (85, 89). Periostin within the lung environment directly interacts with Wnt agonists Wnt1 and Wnt3a to potentiate Wnt signaling and promote the stemness characteristics of disseminated breast cancer cells (85). This supports the survival of these cells and their self-renewal capacity in initiating secondary tumors. Together with its interaction with core matrisomal proteins (Tenascin C, fibronectin, and type I collagen) and ECM remodeling proteins such as lysyl oxidases, the high expression of periostin in lung cancer contributes to the structural and functional ECM network within lung tissue that enhances the metastatic potential of cancer cells (90). Importantly, in breast cancer metastasis models, periostin-mediated tumor initiation is specific to the lung microenvironment, since it does not affect the progression of primary breast tumors (85). For this reason, the pro-metastatic effects of periostin may also play a role in promoting intrapulmonary metastasis of lung cancer and this mechanism warrants investigation.

Thrombospondins

The glycoproteins of the thrombospondin family, primarily thrombospondin-1 and thrombospondin-2, are produced by immune, vascular and stromal cells within the lung and are known to inhibit the initiation of primary tumors and the outgrowth of secondary tumors in the lung (33, 91). One of the major mechanisms by which thrombospondin-1 regulates tumor outgrowth and dormancy is by interacting with the latency associated peptide in complex with TGFβ to release TGFβ into the local environment (92) and thereby stimulate TGFβ signaling (see Tenascin-C above). While this has been demonstrated *in vitro*, it is not clear if thrombospondin-TGFβ interactions regulate dormancy in the lung and at secondary lung cancer sites *in vivo*.

Studies in breast cancer models of lung metastasis have identified that cancer cells with high AXL expression also highly express thrombospondin-2 and this drives TGFβ1-dependent lung colonization (93). Upon colonization, interaction with fibroblasts in the lung triggers a phenotypic switch in the disseminated tumor cells to a proliferative, epithelial phenotype with activation of BMP-dependent SMAD 1-5 signaling, and downregulation of TGFβ-dependent SMAD 2/3

signaling to promote tumor outgrowth (93) (**Figure 1D**). In contrast, thrombospondin-2 expression by lung cancer cells promotes lung cancer bone metastasis through osteoclastogenesis (94) which may explain the association of this isoform with recurrence in adenocarcinoma (95). These data point to a role for thrombospondin-2 in promoting cancer cell proliferation in metastatic foci within the lung, although it is not clear what role this protein plays in primary tumor onset, progression, and evolution.

The role of thrombospondin-1 in lung tumors, however, remains contentious. Serum and intratumoral levels show conflicting associations with patient outcome (95–97), which may reflect differences in the release of thrombospondin-1 from the tumor microenvironment into the systemic circulation, or its specific localized effects within the tumor environment. This discrepancy may also be partly explained by differential effects of thrombospondin-1 on the initiation compared with the progression of lung tumors. Unlike thrombospondin-2, thrombospondin-1 acts as a tumor suppressor in pre-malignant Kras-mutant lung carcinoma, by inducing p53-dependent epithelial cell senescence (98). It is not known if this role for thrombospondin-1 is abrogated in the development of p53-mutant lung tumors, which lack functional DNA damage checkpoints. This may, however, point to a larger genetic dependency for the role of thrombospondin-1 in tumor progression. High MYC expression in breast and prostate cancer cell lines, which mimics MYC amplification seen in a significant proportion of NSCLC tumors, leads to the transcriptional upregulation of prosaponin (99). Prosaponin induces thrombospondin-1 *via* p53 activation in fibroblasts within the tumor environment, thereby increasing thrombospondin-1 levels in the local tumor microenvironment. The context dependency of thrombospondin-1's involvement in lung cancer initiation, primary tumor progression and the establishment of secondary tumors remain to be clarified.

Osteopontin

Osteopontin is a secreted matrix–associated phosphoglycoprotein that is activated by MMP3/7 or thrombin protease (100, 101). High expression of osteopontin is associated with poor survival and tumor aggressiveness in NSCLC (102, 103). Osteopontin interacts directly with α4- and α9β1 integrins to promote tumor cell invasion (104), with the alternatively spliced C-terminally truncated form of the protein being particularly pro-invasive (105). Follistatin-like protein 1, which regulates alveolar maturation in the developing lung by inhibiting BMP4/SMAD1/3/5 signaling (106) and inhibits emphysema development in response to injury (107) also binds directly to pro-osteopontin to prevent its proteolytic activation and thus inhibit lung cancer metastasis. This likely underpins why the expression of follistatin-like 1 protein is downregulated in NSCLC and low expression of this protein is associated with poor survival (101).

Proteoglycans and Glycosaminoglycans

Proteoglycans have a central protein core from which covalently attached linear repeating glycosaminoglycan chains or sulfated polysaccharides extend outward. Proteoglycans are considered a subclass of glycoproteins that specifically contain unbranched and repeating O-linked carbohydrate molecules called glycosaminoglycans. The major glycosaminoglycan classes are hyaluronan, chondroitin, dermatan, heparin and keratan. Proteoglycans are commonly secreted by cells and are enriched in the basement membrane where they act as nucleating components for the fibrillogenesis of collagens and the assembly of elastin fibers (108, 109).

Versican

Versican is a chondroitin sulfate proteoglycan that regulates the perialveolar tissue volume and contributes to alveolar maturation during lung development (110). Although the mechanisms governing versican's role in lung development remain unclear, its interaction with CD44 and β1-integrin is likely to play an important role in these processes. In lung cancer, versican is highly expressed in NSCLC and its expression is tightly correlated with hyaluronan (111). The particular association of versican with patient outcome in adenocarcinoma, but not squamous NSCLC (111) also suggests that its effects may be specific to the progression of the adenocarcinoma histological subtype.

Versican has been shown to be produced by lung cancer cells and activate alveolar macrophages *via* TLR2, TLR6 and CD14 signaling to induce TNFα and IL6 production that subsequently supports lung colonization (112). The association of versican with proliferative signaling in breast, brain, prostate and melanoma cancer cells (113), suggests it may also play a role in regulating the growth of both primary tumors and metastatic foci alike.

Hyaluronan

Hyaluronan is an unbranched heteropolysaccharide glycosaminoglycan that does not form proteoglycans but non-covalently interacts with proteoglycans and other ECM components. It is present within the basement membrane of bronchial and bronchiolar epithelium, within the perivascular region of large blood vessels, and on the cell surface of alveolar macrophages in the healthy lung. CD44, the main cellular receptor for hyaluronan (**Figures 1B,C**), is localized to the basolateral surface of the bronchial epithelia, enabling these cells to bind to hyaluronan in the basement membrane. CD44 expression is increasingly associated with the stemness characteristics of cancer cells, although the presence of cancer stem cells in NSCLC subtypes remains equivocal. At present it also remains unclear whether ECM-CD44 engagement drives stemness phenotypes, or whether the engagement of already upregulated CD44 ligands with ECM components such as hyaluronan preferentially supports cancer cells with stem-like phenotypes. In the normal lung, hyaluronan is expressed by multiple cell types in response to injury, where it is involved in the fibrosis and wound repair response and modulates lung inflammation (114). However, in lung cancer, hyaluronan has been shown to promote the outgrowth of NSCLC tumors (39). During tumor progression, high p38 expression in cancer-associated fibroblasts also induces hyaluronan deposition into the lung tumor environment that subsequently promotes the proliferation and outgrowth of

NSCLC (115). Hyaluronan may also promote the colonization and outgrowth of distant or intrapulmonary metastases, since high hyaluronan levels are associated with increased recurrence rates in lung adenocarcinoma (116). Similarly, high expression of the hyaluronan receptor CD44 on NSCLC cells is associated with squamous histology, as well as increased metastasis and poor survival in adenocarcinoma patients (117). In response to hyaluronan, CD44 receptor engagement activates multiple downstream signaling pathways including activating TGFβ signaling as well as Nanog-Stat2, Oct4-Sox2-Nanog and c-Src-kinase signaling that culminate in STAT3 or CBP/p300 mediated transcriptional reprogramming (including inducing MMP, cyclin-D1, pro-survival, and EMT gene expression) as well as actin cytoskeletal remodeling (**Figures 1C–E**). Together these collectively promote the self-renewal capacity of lung cancer cells, support the survival of cells at the primary tumor site and prime them for metastatic dissemination and colonization (118, 119).

Age-related loss of the hyaluronan and proteoglycan link protein 1 (HAPLN1), which directly binds hyaluronan (120), has also been associated with increased lymph node permeability and increased lymphatic metastasis in melanoma (121, 122). While it is not clear if this mechanism also operates in lung cancer, it reflects broader changes in extracellular matrix secretion by pulmonary fibroblasts seen during aging and paralleled in chronic lung diseases (123), that may explain observed increases in metastatic propensity associated with age in this NSCLC.

MATRISOME-ASSOCIATED FACTORS IN LUNG DORMANCY

Structural matrisomal proteins in the lung extracellular matrix directly interact with soluble growth factors and ECM degrading enzymes (124). Secreted factors specific to the lung are likely to contribute to the lung tropic characteristics of dormancy and outgrowth.

Many proteins that associate with the core matrisome regulate dormancy and outgrowth in a tissue- and context-specific manner. The most prominent of these is TGFβ, which is physically tethered to the matrix and is released upon matrix remodeling. The lung has a uniquely high expression of the TGFβ superfamily member BMP4, which promotes the dormancy of disseminated tumor cells within this tissue (125). In breast cancer models, disseminated cancer cells can overcome the suppressive microenvironment of the lung by expressing Coco, which directly binds to BMP4, preventing the activation of TGF receptors and triggering the re-awakening of dormant cells (125) (**Figure 1B**). Importantly, this mechanism appears to be specific to lung tissue and may explain the lung tropic behavior of non-pulmonary primary tumors. Low endogenous levels of another ligand of the TGFβ superfamily, TGFβ2, in the lungs has also been proposed as a mechanism supporting the outgrowth of disseminated tumor cells and may explain the short latency of metastatic foci in the lungs compared with other organs (126). However, it is not clear if these same mechanisms contribute to the dynamics of primary lung tumors or their intrapulmonary metastases.

The canonical ligands regulating stemness also interact with the extracellular matrix and modulate the metastatic capacity and dormancy phenotypes of cancer cells at primary and secondary sites. Both lung cancer cells and disseminated cancer cells that colonize the lung are enriched for stemness characteristics (127). This stem-cell like population of lung and breast cancer cells are more responsive to collagen remodeling and integrin-mediated reawakening mechanisms (28, 128). Wnt and Notch signaling are fundamental pathways that regulate these stemness characteristics of NSCLC cells and support the survival of disseminated cancer cells in the lung (129–131) (**Figure 1E**). Lung and breast cancer cells with high expression of the Wnt inhibitor DKK1 and the stemness factor SOX2 survive in dormant colonies within the lung, brain and kidneys (28). In this context, high DKK1 and SOX2 expression induces a slow proliferative state and self-renewal phenotype that enables these disseminated cells to persist. Furthermore, DKK1-driven evasion from Natural Killer (NK) cell–mediated clearance by downregulation of NK cell activators supports the maintenance of these dormant colonies (28). High expression of DKK1 also mediates the outgrowth of bone-colonizing lung adenocarcinoma cells by inhibiting osteoblast differentiation in the pre-metastatic niche as well as the induction of the Wnt signaling transcription factor LEF1 and embryonic development gene HOXB9 (132, 133). The interaction of extracellular matrix components with Wnt ligands regulates their availability to induce the proliferation and differentiation of multiple pulmonary cell types as well as lung cancer cells (133).

The association of these signaling effectors with the core matrisome in the lung creates a dynamic functional network that directs the behavior of primary and disseminated cancer cells alike. An improved understanding of how the dynamics of these protein-protein interactions influence cancer cell dormancy and dissemination will generate a more complete picture of the role of the extracellular matrix as a functional, as well as a structural component of the tumor microenvironment. The dynamics of extracellular matrix synthesis and turnover, which regulates the release and sequestration of these matrisome-associated factors, is therefore a significant consideration in the functional dynamics of the tumor microenvironment.

THE DYNAMIC MATRIX AND ITS ECM REMODELING FACTORS

The extracellular matrix is a dynamic component of the tumor microenvironment that regulates the capacity of tumor cells to migrate away from the primary tumor and colonize local or distant sites to form overt metastases. The structure and composition of the extracellular matrix is remodeled by multiple enzymes, such as the lysyl oxidases, MMPs and ADAMTS families that mediate both the crosslinking and degradation of ECM components. The degradation products of these remodeling processes are far from passive byproducts. Many of these products of catabolic ECM remodeling have individual signaling roles that mediate crosstalk between cell types within the tissue environment or are released systemically to influence

the function of multiple organ systems. Therefore the dynamic remodeling by these enzymes significantly contributes not only to the ECM composition within the local tissue environment but also to the functional responses of cells within both the local and systemic environments.

ECM Cross-Linking and Stabilization

Collagen architecture is largely built up by the crosslinking of collagen fibrils to form highly bundled collagen fibers. Collagen crosslinking is initiated by lysyl hydroxylases, which catalyze the intracellular hydroxylation of lysine residues. Lysyl hydroxylase 2 is highly expressed in adenocarcinoma, where it promotes NSCLC metastasis by increasing tumor stiffness to activate cancer cell invasion, PI3K signaling and modulates collagen remodeling (134–136). These studies indicate that although lysyl hydroxylases typically operate on intracellular collagen at the endoplasmic reticulum, lysyl hydroxylase 2 is also secreted into the extracellular compartment of the tumor microenvironment where it modifies collagen fibers (136). It is not yet clear if the intracellular or extracellular activity of lysyl hydroxylase-2 more significantly contributes to its pro-metastatic effect.

Enzymes of the lysyl oxidase family catalyze the oxidative deamination of hydroxy-lysine and lysine to reactive aldehydes once the collagen has been secreted into the extracellular compartment. These reactive aldehydes can then spontaneously crosslink neighboring fibers. This lysyl oxidase-mediated collagen cross-linking is essential to the formation of mature collagen fibrils and fibers that underpin the structural integrity of the collagen matrix. This highly crosslinked collagen is also more resistant to MMP–mediated degradation (137) and the crosslink density is known to determine the stiffness of the lung tissue (138).

In non-pulmonary cancers, the lysyl oxidase family profoundly promotes the dissemination of cancer cells from the primary site as well as the colonization of and outgrowth at distant sites, including the lung, through direct effects on collagen architecture and tissue biomechanics (41, 139–144), and through the recruitment of myeloid cells to the premetastatic niche (68). The secretion of lysyl oxidase (LOX) by tumor cells in response to intratumoral hypoxia, or as a wound healing response to surgery, can increase the systemic LOX activity, thereby catalyzing collagen crosslinking at distant sites, including within the lung (68, 145). This systemically elevated LOX activity can therefore promote metastatic colonization and outgrowth at distant sites. In NSCLC, the expression of LOX and lysyl oxidase-like 2 (LOXL2) are upregulated and increase the metastatic potential of lung tumor cells (144). These enzymes are insufficient to drive cancer cell invasion alone (144) and instead appear to operate in concert with mesenchymal phenotypic programs to facilitate dissemination.

The importance of lysyl oxidases in lung cancer dynamics is dependent on the histological subtype and genomic profile of the tumor. In particular, loss of LKB1, which occurs in 34% of adenocarcinoma and 19% of squamous carcinomas (146), induces mTOR-HIF1α signaling to induce lysyl oxidase expression within the tumor microenvironment. Lysyl oxidase-mediated collagen crosslinking then increases the stiffness of the tumor tissue and activates pro-proliferative β1-integrin signaling (142). Interestingly, increased LOX activity in this context is also associated with increased transdifferentiation of adenocarcinoma cells toward a squamous phenotype (142), suggesting that remodeling of the local ECM and increases in tissue stiffness may contribute to the development of the squamous histological subtype of NSCLC.

ECM Turnover

Proteases play an important role in the degradation and turnover of all matrix components in the lung and at distant sites. In the lung, neutrophil-derived MMP9, cathepsin G and elastase cleave laminin-111 (61) and thrombospondin-1 (147) to promote the outgrowth of cancer cells within the lung. High MMP2 and MMP9 expression are also associated with increased intrapulmonary metastatic potential of lung adenocarcinoma (148). In addition, high MMP13 expression drives the colonization of the brain by lung adenocarcinoma cells, but was not found to functionally influence the growth of these micrometastases after their colonization (8).

Proteases within the lung environment also cleave ECM components to release growth factor ligands that regulate pro-proliferative intracellular signaling events. For example, BMP1 cleaves latency associated binding peptides thereby releasing TGFβ into the microenvironment to promote dormancy (149). Conversely, MMP14 expressed by NSCLC cancer cells and myeloid cells in the tumor microenvironment digests the heparin-binding EGF-like growth factor (HB-EGF) to generate both soluble and membrane-bound heparin-independent growth factors that can activate EGFR signaling (150, 151). EGF-like signaling ligands are also released by MMP14-mediated degradation of the laminin 5 γ2 chain to generate EGF-like fragments that drive EGFR signaling toward increased NSCLC tumor growth (150, 151). Similar MMP14-mediated remodeling operates in the wound healing response of the normal lung to regenerate alveoli (150, 151), supporting the notion that aberrant wound healing responses significantly contribute to disease progression in NSCLC. Amplification of another member of the metalloproteinase family, MMP13, promotes brain metastases specifically in Kras-mutant lung adenocarcinoma (8). Although the precise mechanism underlying this association has not yet been dissected, this does suggest that the role of MMPs in lung tumor dissemination is likely to depend on the genetic profile of the tumor.

Proteases that directly modify core matrisomal proteins also activate other ECM remodeling enzymes. For example, BMP1 which activates fibrillar pro-collagens by removing their N and C-termini, also cleaves the pro-LOX protein to release the mature active enzyme (45). This pleiotropic activity synchronizes extracellular matrix secretion and deposition with its stabilization and remodeling within the microenvironment. Furthermore, the pleiotropic activity of BMP1 and other proteases in releasing growth factor ligands that are either tethered to the extracellular matrix (e.g., TGFβ), or as motifs sequestered within intact protein targets of these proteases (e.g., EGF-like domains), also orchestrates both ECM and growth factor signaling (152), which converge on the same intracellular signaling pathways

(**Figure 1D**). This synchronization of extracellular events and the symmetry of intracellular signaling means that extracellular matrix remodeling can generate profound changes in cancer cell behavior to collectively regulate tumor dynamics by multiple mechanisms.

ECM BIOMECHANICS IN LUNG CANCER PROGRESSION AND DORMANCY

The network behavior of matrisomal proteins determines the biomechanical properties of lung tissue. Elevated fibrillar collagen content and higher levels of crosslinking between collagen fibrils in NSCLC tumors increases the mechanical stiffness of the tissue (138). Mechanosensitive receptors then activate intracellular signaling networks that promote the dissemination of cells away from the primary tumor and support the establishment of intrapulmonary and distant metastases.

The increased stiffness of the lung tumor environment, driven by many of the processes described above, is sensed by lung fibroblasts through a number of mechanisms, including β1-integrin engagement. This mechanosignaling then directs their transition to a myofibroblast-like phenotype that secretes high levels of matrisomal proteins as well as ECM remodeling enzymes (153). This mechanoresponsive signaling creates a self-reinforcing amplification loop that further increases the ECM stiffness in the tumor microenvironment to promote the growth of primary NSCLC tumors (154–156) (**Figure 1E**). In *in vitro* models, this mechanoresponsive fibroblast activation can persist for several weeks once the matrix stiffness is reduced, suggesting that transient stiffening of lung tissue in response to injury may also induce long lasting effects on cellular behavior that can potentiate tumorigenic processes (157). In experimental models of idiopathic pulmonary fibrosis, stiff matrices signal through FAK and Rho kinase in lung fibroblasts to regulate multiple downstream pathways including YAP/TAZ, which converge on apoptotic mediators to inhibit fibroblast apoptosis, as well as further accelerating pro-fibrotic remodeling (158). Overlapping mechanisms are seen in NSCLC, where stiff matrices also drive FAK activation and β-catenin accumulation that increase the responsiveness of intracellular Wnt signaling and drive self-renewal programs in NSCLC cells (159). These effects are also seen in breast cancer models where stiff matrices promote focal adhesion assembly and enhance cytoskeletal tension to increase the activation of ERK and PI3K in response to growth factor ligands in tumor cells (160–162). In pancreatic ductal adenocarcinoma cells, oncogenic Ras activity can further amplify these responses by stimulating ROCK activity to subsequently stiffen the extracellular matrix and drive tumor growth (163), although this is yet to be demonstrated in non-small cell lung cancer where activating Ras mutations are common.

Lung tissue is under constant mechanical stress due to strains induced during normal breathing. On a micro scale, cellular contraction pulls on the extracellular matrix components to also generate strain within the lung. The tensile and compressive strain within the lung extracellular matrix regulates the sequestration and release of matrix-associated factors. For example, TGFβ is tethered to the extracellular matrix by latent TGFβ binding proteins, which interact with fibronectin, fibrillins, fibulins, proteoglycans, and other fibrillar ECM components, as well as the latency associated peptide (LAP), which binds to integrin receptors (73, 81, 82). Mechanical tension induced by cellular contraction, including by highly contractile pulmonary myofibroblasts (164), causes a conformational change in the TGFβ-LAP complex that releases the TGFβ ligand and enables it to activate the TGFβ receptor (73, 81). While this operates at a low level to drive localized lung regeneration in the wound healing response, the severe impairment of lung regeneration in idiopathic pulmonary fibrosis causes extensive alveolar loss that significantly elevates the tensile forces that type II pneumocytes are exposed to (165). This increases the release of TGFβ into the alveolar environment to activate pro-fibrotic TGFβ signaling in lung fibroblasts (165). Because alveolar loss occurs in the periphery, this generates progressive fibrosis from the distal parenchyma toward the central airways, that is a feature of idiopathic pulmonary fibrosis progression. This newly described mechanism raises the possibility that altered force distribution throughout the lung as a result of perturbed ECM remodeling in lung tumors or in chronic lung diseases, including COPD, may also induce TGFβ signaling to support tumor dormancy or, conversely, a pro-fibrotic environment that drives cancer cell proliferation. This mechanism may partly explain why IPF patients are at increased risk of developing lung tumors and why these tumors develop close to the fibrotic regions in IPF lungs (24, 166, 167).

Integrin signaling is responsive to mechanical strain within lung tissue, which occurs during normal breathing and is perturbed by ECM remodeling. In addition to having different affinities for different ECM components, integrin receptor subtypes display different temporal kinetics in response to mechanical force, allowing them to activate different mechanotransduction pathways in response to specific changes in lung biomechanics (168). For example, cyclic strain, which mimics breathing biomechanics, induces gefitinib resistance *via* β1-integrin signaling in EGFR mutant lung adenocarcinoma (169). Integrin clustering also mediates downstream signaling diversity, while stress-induced conformational changes in focal adhesion proteins, such as talin, can fine-tune the response of cells to the magnitude of local forces (170). Moreover, the biomechanics of the tumor microenvironment feed back onto the architecture of the extracellular matrix itself. For example, fibronectin assembly and collagen fibrillogenesis are both regulated by tension applied to fibronectin (65, 109, 171). In this way the lung biomechanics create positive and negative amplification loops that can accelerate the outgrowth of primary and disseminated tumor cells or drive tumor cells into sustained dormancy.

STROMAL CELLS AND THEIR ROLES IN DORMANCY AND OUTGROWTH

Fibroblasts

As the major producer of extracellular matrix components, lung fibroblasts play an important role in regulating the lung ECM in

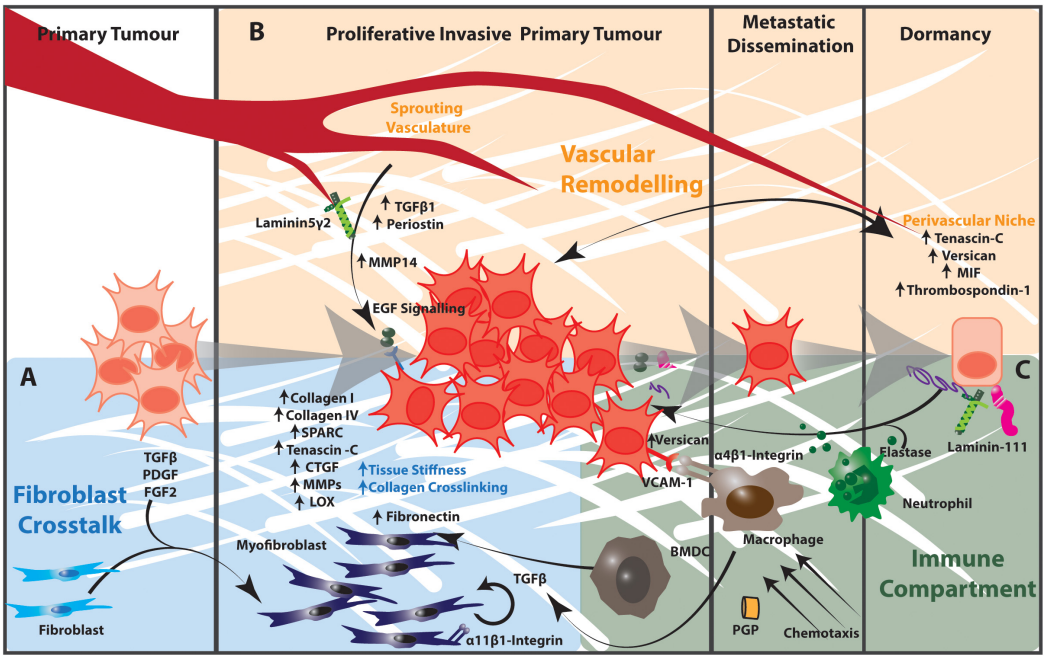

FIGURE 2 | The tumor microenvironment contributes to matrix remodeling that regulates tumor cell dormancy and outgrowth in NSCLC. **(A)** Cancer cells release TGFβ, PDGF, and FGF2, which activate fibroblasts to a myofibroblast state. Activated myofibroblasts in the tumor microenvironment produce increased amounts of collagen I, collagen IV, Tenascin C, Thrombospondin-2 as well as remodeling enzymes and signaling effectors such as CTGF and MMPs. The resulting increased matrix stiffness further activates fibroblasts in a TGFβ-dependent manner. Stromal cells expressing α11β1-integrin engage with this extracellular matrix to promote the proliferation, migration, invasion and survival of cancer cells in the tumor microenvironment. **(B)** The extracellular matrix components of the perivascular niche support dormancy in cancer cells. The sprouting vasculature produces TGFβ and periostin that promote cancer cell proliferation and metastatic potential. Endothelial cells of the sprouting vasculature also produce MMP14, which cleaves laminin 5 γ2 to release EGF-like fragments that activate EGF signaling in cancer cells. **(C)** Activated infiltrating macrophages secrete TGFβ into the tumor microenvironment to activate fibroblasts and TGFβ-dependent signaling in cancer cells. α4β1-Integrin-expressing macrophages engage with VCAM-1 expressing cancer cells to induce pro-survival Akt-PI3K signaling. Proteases produced by neutrophils and other immune cells cleave ECM components including elastin and thrombospondin-1. These cleavage products awaken dormant cancer cells and act as chemotactic agents to recruit immune cells into the tumor microenvironment. Collectively the stromal and immunological components of the tumor microenvironment regulate cancer cell dormancy and outgrowth in the lung. BMDC, bone marrow-derived progenitor cell.

both health and disease. The lung fibroblast population is highly heterogeneous and the aberrant expansion of specific fibroblast phenotypes contributes to ECM remodeling in lung tumors. In addition to the effects of increased matrix stiffness discussed above, growth factors such as TGFβ, PDGF and FGF2 secreted by cancer cells and tumor-infiltrating immune cells recruit and activate fibroblasts to a myofibroblast-like state (**Figure 2**). This state is characterized by transcriptional and signaling programs that promote fibroblast proliferation, inhibit apoptosis and drive ECM remodeling leading to increased mechanical stiffness. This increased matrix stiffness within the tumor microenvironment creates a TGFβ-dependent amplification loop that further increases both ECM stiffness and fibroblast activation (172–174). These activated fibroblasts also secrete large amounts of collagen I, collagen IV, extra domain A-fibronectin, heparin sulfate proteoglycans, secreted protein acidic and rich in cysteine (SPARC), tenascin–C, thrombospondin-2, connective tissue growth factor, MMPs and plasminogen activators. These each contribute to the significant remodeling of the matrix in the primary lung tumor microenvironment and subsequently alter the behavior of tumor and neighboring untransformed epithelia (93, 172, 174–177).

Multiple transcriptional programs have been identified that distinguish cancer-associated fibroblasts from healthy lung fibroblasts in NSCLC (40, 174, 178). These transcriptional signatures are associated with poor prognosis in NSCLC and center on genes that modulate cell-ECM interactions (40, 174, 178). In addition, increased matrix stiffness due to higher levels of collagen crosslinking within the tumor activates stromal α11β1-integrin signaling to promote the proliferation and increase the metastatic potential of NSCLC cancer cells (154). Emerging evidence indicates that the somatic mutational profile of pancreatic cancer cells can contribute to these phenotypic perturbations by re-educating their surrounding stroma and tissue architecture through paracrine mechanisms to further promote tumor aggressiveness (179). It remains to be determined if particular mutational profiles in non-small cell lung cancer cells drive the specific reprogramming of nearby resident lung fibroblasts in a similar way to indirectly support tumorigenesis.

The metabolic activity of cancer-associated fibroblasts has also been recognized as a means of regulating ECM-dependent dormancy in addition to more established bioenergetic co-dependencies between these cell types (180). Cancer-associated fibroblasts have increased glycolytic and autophagic activity

compared with normal lung fibroblasts, and have been shown to promote rapid cancer cell proliferation as well as maintain cancer-associated fibroblast survival (27, 180). Since collagen turnover is partially regulated by autophagic proteins (181), the increased autophagic activity of cancer-associated fibroblasts may support more rapid collagen remodeling and an increasingly dynamic extracellular matrix framework. Furthermore, as autophagy activation integrates quiescence and survival signals (27), it is plausible that collagen fibrillogenesis may reciprocally influence autophagy activity to regulate cell survival and dormancy in the lung.

Finally, the long range stiffness gradients generated by fibroblast-mediated ECM remodeling (182) may contribute to field of cancerization effects within the lung, which may promote intrapulmonary metastasis. Crosstalk between epithelial cells and fibroblast subtypes within different lung compartments would generate location-specific regulatory loops that specify the dormancy and outgrowth behavior of tumor cells in a spatial manner. How these processes might contribute to the heterogeneity within and between tumors has not been elucidated. An improved understanding of spatial cues within and between lung compartments may reveal cell extrinsic mechanisms that dictate the emergence of different primary lung cancer subtypes in specific anatomical locations, the character of heterogeneous regions within individual primary tumor masses as well as the location of metastatic foci within the lung.

Immune Cells

Inflammation plays a major role in lung tumorigenesis and etiology (183) and emerging evidence points to extracellular matrix remodeling as both a consequence and instigator of inflammatory processes within the lung. While a comprehensive understanding of the immunological landscape of the lung during the metastatic dissemination, dormancy and reawakening is lacking, emerging evidence is revealing that immune cells interact with the extracellular matrix to contribute to these processes. The different immunological landscapes associated with specific oncogenic driver mutations in NSCLC (184) may also contribute to the heterogeneity in extracellular matrix remodeling during tumor progression.

Immune cells can re-educate resident fibroblasts in and around tumors, and likely play an important role in establishing a permissive environment that supports lung colonization and outgrowth. In addition, immune cells are capable of synthesizing extracellular matrix proteins, although they not a major source of extracellular matrix components within tissues. For example, myeloid cells produce versican (185) while activated and infiltrating macrophages secrete TGFβ to induce the pro-fibrotic transformation of resident fibroblasts. Immune cells also remodel extracellular matrix proteins to regulate cancer cell dormancy and the local immune landscape, while the proteolytic products of these reactions also recruit and activate other immune cells. For example, as discussed above, neutrophil–derived proteases such as elastase cleave laminin-111 and thrombospondin-1 to awaken dormant breast cancer cells within the lung (61, 147), and the cleavage products of elastin are highly chemotactic for monocytes (**Figure 2**) (186). Furthermore, the degradation product of type I collagen, Proline-Glycine-Proline tripeptide (PGP), mimics CXC chemokines such as IL-8 and interacts with the CXCR1 and CXCR2 receptors to attract neutrophils to the ECM remodeling site (187). This further amplifies extracellular matrix remodeling within the tumor microenvironment and regulates the proliferative behavior of tumor cells in this environment.

These tumor infiltrating immune cells also directly interact with disseminated tumor cells in the lungs to regulate their proliferative state through ECM responsive integrin and TGFβ signaling. Activated α4β1 integrin-expressing macrophages associate with VCAM-1 positive cancer cells in the lung to induce pro-survival Akt-PI3K signaling in tumor cells (188). Similarly, bone-marrow derived progenitors recruited to the premetastatic lung induce the mesenchymal to epithelial transition of tumor cells through downregulation of SMAD2 signaling in the canonical TGFβ pathway, and a switch to macrometastatic growth (185). Characterization of the NSCLC immune landscape will further define how complex interactions between these immune cells, cancer cells and stromal cells within the tumor microenvironment modulate the extracellular matrix, and subsequently the dynamics, of primary and secondary NSCLC tumors.

Vascular Architecture and Dormancy

The dynamics of the pulmonary capillary also regulates tumor cell dormancy and outgrowth, although primarily in the context of metastasis to the lung from non-lung primary tumors. Once primary tumor cells have disseminated from the primary tumor site, contact with the epithelial basement membrane is replaced by binding to the endothelial basement membrane that surrounds capillaries. In metastatic models of primary lung tumors, the metastatic colonization of distant sites involves extravasation of lung tumor cells at vascular branch points and the outgrowth of tumors at these perivascular locations (189). While the physical size of the capillary bed may play a role in physically trapping disseminated tumor cells in the pulmonary capillary bed or at vascular branches in distant organs, this perivascular niche that surrounds the vasculature also presents specific cues that regulate the proliferation of lung epithelia and disseminated tumor cells alike (151). The destabilization of pulmonary vasculature occurs early in NSCLC as well as in breast cancer metastasis to the lung (190–192), supporting the notion that remodeling of the perivascular niche significantly contributes to tumor progression.

In breast cancer metastasis, endothelial tip cells within the perivascular niche deposit increased levels of periostin, tenascin-C, versican, S100 proteins, TGFβ and MIF, which act to maintain cancer cell dormancy (**Figure 2**) (193). Non-sprouting endothelial cells also produce thrombospondin-1 which associates with the mature microvascular basement membrane to sustain quiescence (89). Conversely, the production of TGFβ1 and periostin by tip cells of the sprouting neovasculature can promote tumor outgrowth (89). In this way cancer cells become dormant in the presence of dormant microvasculature, and become reactivated during angiogenic sprouting through interactions with the extracellular matrix of the perivascular niche. Furthermore, VEGF and FGF signaling in pulmonary endothelial cells induces MMP14 expression, which releases

EGF-like ligands from the laminin 5 γ2 chain and drives pro-proliferative EGF signaling in epithelial cells (151). Together these mechanisms link the remodeling of the extracellular matrix within the perivascular niche to the proliferative behavior of disseminated tumor cells.

EXPLOITING THE TUMOR ECM TO IMPROVE CLINICAL PRACTICE

Using the ECM to Inform Clinical Decision Making

The extracellular matrix features that characterize NSCLC patients at high risk of poor outcome outlined above have the potential to be utilized as clinical decision-making tools in determining the most effective therapy for a patient's condition. In NSCLC, where there are currently no clinicopathological features identifying early-stage patients at high risk of disease recurrence following surgery, there is a clear need to identify the distinguishing characteristics of this population. Transcriptional signatures associating matrisomal gene expression with lung cancer recurrence holds promise that such approaches may robustly identify early stage patients at high risk. This will become more important as early stage diagnoses continue to increase (11). Improvements in treating NSCLC will require an understanding of the burden of disseminated tumor cell dormancy at diagnosis, as well as the relative threat posed by the likely presence of metastatic niches and dormant disseminated tumor cells in terms of their ability to grow into macrometastases, or alternatively remain dormant for many decades.

The substantial progress in understanding the extracellular matrix dynamics occurring during primary tumor development and metastatic colonization of many tumor types to date provides hope that this knowledge may improve clinical practice in the future. Although, our understanding of these processes in lung cancer is lagging behind that of other common cancers, such as breast cancer. Of critical importance in realizing this goal will be developing biomarkers of these processes that perform with satisfactory sensitivity and specificity. Moreover, it remains a challenge to develop tests that are capable of extending beyond robust performance on population level data to predict risk for an individual patient. Such readouts must also be readily appropriated to existing clinical management pipelines.

This understanding could also establish a path to develop therapies targeting tumor cell dormancy in NSCLC. Dormant cells largely escape immune surveillance and are resistant to conventional and targeted chemotherapies (194). Therefore, strategies to treat metastasis by targeting tumor dormancy follow two main approaches (33): (1) trap disseminated tumor cells in a dormant state in the long term or (2) reawaken dormant cells into a proliferating state and eradicate them using standard of care treatments. Distinguishing between patients where dormant cells are at high risk of becoming reactivated, for example by extracellular matrix remodeling in response to injury or environmental exposure, compared with those that may remain dormant indefinitely, is key to effectively managing cancer as a chronic and ultimately curable disease.

A deeper understanding of the role of the tumor microenvironment will also assist in determining which existing therapies are the most appropriate for the patient. It will also be important to determine whether surgical resection in early stage cancers is likely to increase the risk of activating already disseminated tumor cells and therefore worsening the patient prognosis (145). Similarly, chemotherapy- or radiotherapy-induced lung fibrosis may also accelerate the awakening of otherwise dormant cancer cells in the lung (195). Some conventional chemotherapy agents that induce DNA damage may also activate quiescent cells to re-enter the cell cycle (33) and may be preferred in patients suspected to have a high burden of dormant disseminated tumor cells. Conversely, existing therapies that are effective at inducing dormancy in disseminated cells may be used as maintenance therapies to keep these cells in a dormant state (34, 196, 197). For example, the EGFR targeted therapy erlotinib in combination with a BH3 mimetic induced prolonged quiescence in preclinical models of NSCLC xenografts (198), suggesting that therapeutic approaches that achieve disease control in the long term may require combination therapies that simultaneously inhibit mitogenic and apoptotic signaling pathways. Whether these approaches can be applied in the stromal targeting context remains to be seen.

However, consideration must be given to the impact of such therapies on stromal cells within the tumor microenvironment. For example, cisplatin treatment induces AXL and GAS6 expression by cancer associated fibroblasts to promote the migration of AXL-expressing lung cancer cells (199). An increasing number of clinical trials testing the ability of conventional therapies to target disseminated tumor cells as primary endpoints of their clinical trials (200) coupled with technological advancements to detect disseminated tumor cells, will be critical to facilitate the testing of more ECM-centric targeted therapies that reawaken or eradicate dormant tumor cells.

Novel Therapeutic Targeting of the ECM

Recent advances in our understanding of how the matrix is impacting tumor cell dormancy has also led to the development of novel therapeutic strategies aimed at maintaining dormancy or eradicating dormant cells.

The recognition of common fibrotic mechanisms in idiopathic pulmonary fibrosis and lung cancer has led to interest in repurposing anti-fibrotic IPF therapies to the treatment of lung cancer. Many anti-fibrotic therapies have met limited success in clinical trials for their ability to inhibit metastatic colonization and promote dormancy maintenance. However, the VEGF, PDGF and FGF inhibitor Nintedanib, which is used in the treatment of idiopathic pulmonary fibrosis, has been approved in some jurisdictions as a second line therapy in combination with conventional chemotherapy (e.g., pemetrexed) in NSCLC and renal cell carcinoma (201). By blocking the activation of fibroblasts to myofibroblasts, it significantly reduces the degree of ECM remodeling within tumors. Pirfenidone, the other approved anti-fibrotic IPF therapy, is still being tested for its efficacy as a NSCLC treatment. Other IPF treatments targeting galectin-3 (TD139), TGFβ (GC1008), αvβ6 integrin signaling (BG0011, GSK3008348) and inflammatory mediators (QAX576, Carlumab)

are currently under clinical development for IPF but may yet find utility as NSCLC therapies (24). The lack of treatments targeting mechanisms underlying COPD precludes repurposing of these agents into the oncology space, although it is hoped that emerging treatments for COPD may also prove efficacious in treating lung cancer patients.

Additionally, LOX-neutralizing antibodies that block collagen I deposition and cross-linking have shown utility in preclinical studies targeting metastasis of non-pulmonary tumors to the lung (68, 143). Preclinical development of LOX inhibitors for non-pulmonary tumors are ongoing, although their efficacy in NSCLC remains untested. Antibody targeting of LOXL2, which is the other member of the lysyl oxidase family that is highly expressed in NSCLC, have yielded disappointing results in clinical trials in pancreatic and metastatic colorectal cancer (202, 203) but, like LOX inhibitors, may yet prove effective in NSCLC treatment.

Directly targeting the extracellular matrix components themselves has also shown promise. Antibodies against the pro-proliferative proteolytic fragment of laminin-111 suppressed the outgrowth of breast cancer metastases in the lungs in a preclinical model (61), although its efficacy in primary lung tumors has not yet been tested. Glycoproteins or short peptides derived from quiescence-inducing proteins such as the thrombospondin-1-inducing glycoprotein prosaposin have successfully induced systemic thrombospondin-1 to inhibit the metastatic outgrowth of prostate and breast cancer lung metastases (91, 99). However, due to the contentious role of thrombospondin proteins in NSCLC progression, it is not yet clear whether this approach will also be effective in inhibiting the growth of primary lung tumors or their intrapulmonary metastases.

Other experimental approaches are targeting key signaling processes downstream of matrix engagement. Suppression of MAPK signaling, or by targeting EGFR, MMP9, amphiregulin, FAK, SRC kinases, ROCK or PI3K, as well as by sustaining or inducing p38 and HOXD10 signaling have proven effective at inducing dormancy in breast and pancreatic cancer models (193, 204) and similar approaches may be effective in NSCLC. Targeting MLCK to inhibit actin remodeling that switches cells from dormancy to proliferation in response to the extracellular matrix is also being explored in breast cancer and osteosarcoma models that metastasize to the lung (49). α5β1- and α5β3- integrin targeting antibodies, cyclic peptides or peptidomimetics have also been trialed to suppress lung colonization and outgrowth in breast cancer metastasis but have yielded disappointing clinical trial results (53). Similarly, integrin-targeting antibodies are being developed as PET imaging tracers for the diagnosis of cancer (205).

Other approaches are using matrix component epitopes to target drugs directly to the tumor site. For example, the collagen-binding properties of lumican have been exploited to target collagen-rich melanoma tumor environments with interleukins (206) and may be applicable to the high collagen content of NSCLC tumors. Similarly, immune checkpoint therapies conjugated to a heparin binding domain peptide with a high affinity for glycoproteins and some collagen proteins have also shown promise in preclinical studies in melanoma and breast cancer models (207). Matrix-targeting nanobodies are also being developed as PET tracers which may be adapted to mediate ECM-targeted drug delivery (208). The diverse and complementary approaches being pursued to exploit the role of the extracellular matrix in tumor dormancy and outgrowth provide promise that effective therapies will be developed to manage NSCLC cancer as a chronic disease.

FUTURE CHALLENGES

While tremendous progress has been made in understanding the complex interplay between cancer cells, the extracellular matrix and the surrounding stromal cells in regulating the dynamics of primary and secondary tumors in many cancer types, the interrelated functions of these components in lung cancer are still being revealed. The complex interplay of ECM components and cell types within the healthy lung, coupled with the highly heterogeneous landscape of lung tumors themselves make this particularly challenging. Defining the phenotypic plasticity of these cell types in healthy and diseased tissue will be fundamental to determining how crosstalk between these cell types orchestrates the extracellular matrix composition of primary and secondary lung tumors. This is particularly the case for fibroblasts and epithelial cells, where lineage-tracing experiments will be invaluable in revealing the extent to which different cell types assume the myofibroblast phenotype in the tumor microenvironment or pre-metastatic niche. Furthermore, as a load-bearing organ, consideration should be given to the mechanical cues that regulate cell behavior within these lung compartments.

The development of *in vitro* and *in vivo* models that more accurately recapitulate the dynamic ECM remodeling in these tumors will be key to dissecting the contribution of individual cell types and matrix components to the broader network. In particular, robust models of the squamous NSCLC subtype and of metastatic processes in NSCLC more generally are currently lacking. Importantly, directly comparing these models and the genomic features associated with them will clarify the context-dependent cues that are currently preventing the development of an integrated model of these processes. It is not currently clear how quickly the ECM is remodeled within the healthy or diseased lung, how long the remodeled matrix persists in a given state, or the temporal hierarchy of how these ECM features evolve over time. An understanding of how early ECM remodeling occurs in the initiation of lung tumors, and whether this tumor-associated ECM remodeling persists following remission will be fundamental to understanding the temporal dynamics of lung tumor progression and relapse.

Our current understanding has identified the impact of ECM composition at the level of individual ECM components. However, the ECM acts a three-dimensional network of matrisomal proteins together with matrisome-associated factors and signaling effectors. An improved understanding of the role of the ECM in regulating dormancy and outgrowth will rely on clearly defining the protein-protein and protein-carbohydrate interactions that form these networks and how individual nodes manipulate the collective three-dimensional architecture of the ECM. High-resolution optical imaging and spatial proteomic

technologies to map these interactions will be invaluable in defining these spatial relationships.

Historically, the late presentation of NSCLC has obscured our understanding of early stage disease processes. Furthermore, there is currently no way to establish when tumor cell dissemination establishes intrapulmonary and distant dormant micrometastases to facilitate the direct study of the ECM in these events. However, improved early detection strategies are increasing the proportion of patients diagnosed with early stage disease, and thereby providing opportunities to gain insight into these early dynamics. The analysis of these early stage tumors using improved sequencing and proteomic technologies will also shed light on the true incidence and clonal evolution of the primary and metastatic tumors. Accurate determination of the incidence of intrapulmonary metastases and the contribution of ECM-driven processes to recurrence following surgery will be fundamental to improving patient outcome in early stage disease. Similarly, an understanding of how extracellular matrix remodeling in chronic lung diseases such as chronic obstructive pulmonary disorder and idiopathic pulmonary fibrosis affects the transformation of lung epithelia and the progression of established NSCLC tumors may partly explain why these conditions are associated with an elevated risk of developing these tumors. Inhibition or reversal of ECM remodeling in these fibrotic diseases may be a crucially important approach to prevent lung cancer initiation in these patients.

Taken together, these developments in understanding the structural and functional role of the ECM in NSCLC progression will reveal novel potential therapeutic strategies that are able to identify patients at high risk of developing recurrent disease as well as the optimal therapeutic approaches based on both the cell-intrinsic and -extrinsic mechanisms operating within the tumor microenvironmental rather than focusing on cell-intrinsic somatic genomic alterations alone. Novel therapeutic approaches that sustain disseminated cells in a dormant state, or awaken and eradicate the residual disease, will see lung cancer become a manageable chronic illness and ultimately a curable condition.

AUTHOR CONTRIBUTIONS

AP and TC contributed equally to the conception, writing, and reviewing of this manuscript. Both authors contributed to the article and approved the submitted version.

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Glioma Cells With Genetically Engineered IGF-I Receptor Downregulation Can Persist in the Brain in a Dormant State

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Glioblastoma multiforme is an aggressive malignancy, resistant to standard treatment modalities and associated with poor prognosis. We analyzed the role of the IGF system in intracerebral glioma growth using human and rat glioma cells. The glioma cells C6 and U87MG were transduced with a genetically engineered retrovirus expressing type 1 insulin-like growth factor (IGF-IR) antisense RNA, either before or after intra-cerebral implantation of the cells into Sprague Dawley rats or nude mice, respectively and tumor growth and animal survival were monitored. Rat glioma cells transduced prior to orthotopic, intra-cerebral implantation had a significantly increased apoptotic rate *in vivo* and a significantly reduced tumor volume as seen 24 days post implantation ($p < 0.0015$). This resulted in increased survival, as greater than 70% of the rats were still alive 182 days after tumor implantation ($p < 0.01$), as compared to 80% mortality by day 24 in the control group. Histomorphology and histochemical studies performed on brain tissue that was obtained from rats that survived for 182 days revealed numerous single cells that were widely disseminated throughout the brain. These cells expressed the β-galactosidase marker protein, but were Ki67negative, suggesting that they acquired a dormant phenotype. Direct targeting of the C6 cells with retroviral particles *in vivo* was effective and reduced tumor volumes by 22% relative to controls. A significant effect on tumor growth was also seen with human glioma U87MG cells that were virally transduced and implanted intra-cerebrally in nude mice. We observed in these mice a significant reduction in tumor volumes and 70% of the animals were still alive 6 months after tumor implantation, as compared to 100% mortality in the control group by day 63. Our results show that IGF-IR targeting can inhibit the intracerebral growth of glioma cells. They also suggest that IGF-IR expression levels may determine a delicate balance between glioma cell growth, death and the acquisition of a dormant state in the brain.

Keywords: glioma, gene therapy, IGF, dormancy, signaling

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common neuroectodermal tumor and the most malignant of cerebral astrocytic gliomas. Despite multimodal treatment regimens, the prognosis for the majority of glioblastoma patients remains poor, with a median survival of less than 1 year (1). There is therefore an urgent need for new therapeutic approaches.

The receptor for the type 1 insulin-like growth factor (IGF-IR) has been implicated in the acquisition of the transformed phenotype and identified as a positive regulator of cancer cell survival, growth and metastasis in a range of tumor types [reviewed in (2, 3)]. In many human malignancies, including GBM, upregulated expression of IGF-IR, IGF-I, IGF-II or combinations thereof have been documented (2, 4). Therefore, targeting the IGF system, by inhibiting ligand or receptor synthesis and/or function could provide effective therapeutic approaches to the treatment of GBM (5–9).

Viral vectors have generally been the vehicles of choice for the delivery of genetic information into tumor cells. Retrovirus-based vectors integrate selectively into actively dividing cells and are thus particularly suited for cancer gene therapy. Pseudotyped viral particles such as VSV-G expressing retro and lentiviruses were used effectively for gene delivery *in vivo* (10, 11).

We produced and evaluated a replication defective MMLV retroviral vector (vLTR-IGF-IR^{AS}) in which an IGF-IR antisense fragment was expressed in a bi-cistronic RNA with EGFP, as we previously described (12). We assessed the anti-tumorigenic potential of this vector using orthotopically implanted human U87MG-LacZ and rat C6-LacZ cells. We found that in addition to causing extensive apoptosis, the downregulation of IGF-IR expression also induced a state of prolonged growth arrest in some of these, otherwise highly aggressive, glioma cells. The results suggest that IGF-IR levels in these cells may regulate a balance between cell growth, apoptosis and the acquisition of a dormant state.

MATERIALS AND METHODS

Cell Lines

Rat glioma cell line C6/LacZ was from American Type Culture Collection (ATCC). The human glioma cell line U87 MG was obtained from the ATCC and transfected with a β-galactosidase expressing plasmid as previously described (13). The 293GPG retroviral packaging cell line (14) and the pLTR-GFP cells were maintained as previously described (15). All cells were routinely tested for mycoplasma and common rodent pathogens.

Production of Retroviral Particles and Viral Transduction

The protocol used to construct the pLTR-IGF-IR^{AS} retrovector was previously described in detail (12). Retroviral particles were produced in the 293GPG packaging cell line and tittered as previously described (16). The pLTR-GFP packaging cell line was used to produce the control viral particles that express the EGFP gene only, using the same protocol. To virally transduce the C6/LacZ cells, 2–4 $\times$ 10^4 cells/well were plated in a 6-well plate and after an overnight culture, 4 $\times$ 10^4 vLTR-IGF-IR^{AS} or control pLTR-GFP retroviral particles were added per well on two consecutive days. The transduced cells were then sorted using a fluorescence activated cell sorter (FACSVantage, Becton Dickinson) and highly fluorescent cells (5–10% highest fluorescent intensity) were selected, designated C6/LacZ^{AS} and C6/LacZ^{GFP}, respectively, and used for all the experiments. The same strategy was used to generate U87MG-LacZ^{AS} and U87MG-LacZ^{GFP} cells.

RT-PCR Analysis

Five microgram of total RNA from each cell line were reverse transcribed using a primer corresponding to nt488-464 of mouse IGF-IR mRNA (100% homology; GeneBank accession # AF056187). PCR was performed using the same primer and a primer corresponding to nt89-113 of mouse IGF-IR. The cDNA products were electrophoresed on a 1% agarose gel, and the bands analyzed by densitometry using the ALPHAImager 2000 software (Alpha Innotech Corporation, San Leandro, CA, United States). The L19 signal was used to normalize the data (17).

Western Blotting

Type 1 insulin-like growth factor receptor levels were analyzed by Western blotting performed as described in detail elsewhere (12) and using a rabbit anti-IGF-IR antiserum (C-20, Santa Cruz Biotechnology, Dallas, TX, United States) and a horseradish peroxidase-conjugated donkey anti rabbit IgG antibody (GE Healthcare Life Sciences, Pittsburgh, PA, United States). To normalize for loading, the membranes were stripped and re-probed with a monoclonal anti-tyrosine tubulin antibody (Sigma-Aldrich, St. Louis, MO, United States). To analyze ERK1 and 2 phosphorylation in response to IGF-I, tumor cells, cultured overnight in serum-free medium, were stimulated with 100 ng/ml IGF-I (US Biological, Salem, MA, United States) for 10 min, lysed on ice in the presence of phosphatase inhibitors and the cell lysates separated on 8.5% SDS-polyacrylamide gels. The blots were probed, first with an anti-phospho-p44/42 ERK (Thr202/Tyr204) antibody and then with an anti p44/42 ERK antibody (Cell Signaling, Whitby, ON, Canada).

Functional *in vitro* Assays for IGF-I Responsiveness

Insulin-like growth factor-I induced cell proliferation was measured by the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Thiazolyl blue] assay and cell survival in serum-depleted medium was measured using propidium iodide (PI) staining (12, 18).

Tumor Cell Growth in Three-Dimensional (3D) Spheroids

Anchorage-independent tumor cell growth was analyzed using spheroids generated from hanging drops, as was previously described (19). Briefly, confluent cultures of C6 cells in 10% FCS DMEM were dispersed by trypsin digestion and the cells

resuspended in DMEM. Twenty microliter drops containing viable cells were placed on the lids of 100 mm culture dishes, which were then inverted over dishes containing 10 ml DMEM and incubated at 37°C for 30 days. The cellular aggregates were then harvested using a Pasteur pipette under a dissecting microscope, placed in 100 mm culture dishes pre-coated with 0.75% agar and overlaid with 10 ml DMEM. The spheroid surface areas were calculated using an inverted microscope equipped with an ocular grid.

Brain Tumor Growth and Apoptosis *in vivo*

All animal experiments were performed in strict adherence to the McGill University Animal Care committee guidelines. Tumor cell implantation was performed as previously reported (13, 16). Briefly, 10^4 rat, or 10^5 human glioma cells were injected intra-cranially into the caudate of adult Sprague Dawley rats or nude mice, respectively, in a stereotactic apparatus (Kopf, Tujunga, CA, United States). Tumor volume measurements were performed on coronal (10 μm) sections stained histochemically for β-galactosidase activity, as described, and the volumes calculated as length (mm) × the square of the width $(mm)^2$ × 0.4. For long-term survival studies, the animals were monitored from the day of tumor implantation until the onset of morbidity and euthanized, as per the McGill University's guidelines for animal care. Apoptotic cells were visualized based on the incorporation of biotinylated dUTP into nicked DNA that was detected by incubation with Cy3-streptavidin (Jackson Lab).

Delivery of Viral Particles *in vivo*

C6/LacZ cells were implanted as described above. Six days later, rats were anesthetized and vLTR-IGF-IR^{AS} or vLTR-GFP (concentrated stock of 3×10^8 cfu/ml) were injected into six different sites (1 mm apart) in the pre-established tumor, guided by the previous stereotactic coordinates. A total volume of 9 μl were injected into each tumor (6 × 1.5 μl increments), and the needle left in place for at least 5 min/increment (for a total of 30 min/tumor). This procedure was repeated one week later. Rats were euthanized 8 days after the last injection. Fluorescence microscopy was used to detect GFP expression in frozen brain sections. Subsequently, sections were stained histochemically for β-galactosidase activity and tumor volumes calculated based on these measurements.

Immunofluorescence

Cryostat sections were air-dried for 30 min, fixed with 4% paraformaldehyde and non-specific staining blocked using 5% normal serum derived from the same species as the secondary antibody. The sections were then incubated overnight at 4°C with the primary antibodies at dilutions of 1:10 (mouse monoclonal anti-Ki-67, Chemicon, Temecula, CA, United States) or 1:100 (rabbit-anti-IGF-IR, Santa Cruz, Santa Cruz, CA, United States), FITC conjugated rabbit anti-β-galactosidase (Rockland, Gilberstville, PA, United States) or mouse monoclonal anti-GFP (Chemicon, Temecual, CA, United States) all in PBS containing 0.1% Trition X-100, 0.1% BSA and 0.1% normal serum.

This was followed by a 1 h incubation at RT with Alexa Fluor 568 goat anti-mouse IgG (Molecular Probes, Burlington, ON, Canada) for detection of Ki-67 and GFP or with Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR, United States) and Cy-3 conjugated donkey anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, United States) for detection of IGF-IR, all at a dilution of 1:200.

Statistical Analysis

The one or two-sided Student's *t*-test were used to analyze differences observed in tumor volumes or in functional *in vitro* assays, respectively. The Kaplan–Meier survival curve and the Log-Rank test were used with Statistica software to analyze survival data.

RESULTS

Enhanced IGF-IR Expression at the Invasive Margins of C6 Tumors Growing in the Brain

We first assessed the expression of IGF-IR in GFP$^+$ C6 glioma cells that were implanted into the caudate region of the rat brain, using IHC. IGF-IR expressing cells were observed throughout the tumor area. However, the intensity of fluorescence was highest at the invading margins of the tumors, underlining the preferential localization of IGF-IR-expressing cells at sites of active invasion (**Figure 1**). In contrast to the region-specific pattern of IGF-IR expression, GFP staining was uniform throughout the tumor mass (**Figure 1**), indicating that the increased intensity of IGF-IR at the tumor margins was not due to a greater concentration of viable cells in this region, or to increased cell death in the inner mass of the tumor. To inhibit IGF-IR signaling during intracerebral tumor growth, we then used a retroviral vector expressing IGF-IR antisense to downregulate receptor expression.

Reduced IGF-IR Levels, Suppressed IGF-I Responses and Impaired Intra-Cerebral Growth of C6 Cells Transduced With vLTR-IGF-IR^{AS} Particles

Sublines C6/LacZ^{AS} and C6/LacZ^{GFP} were generated by transduction of C6/LacZ cells with vLTR-IGF-IR-antisense (vLTR-IGF-IR^{AS}) and vLTR-GFP (vLTR-GFP)-expressing retroviral particles, respectively. Gene transfer efficiency in these cells was 45 and 80%, respectively as revealed by flow cytometry analysis. Highly fluorescent C6/LacZ^{AS} cells (top 5–10 percentile) were enriched by FACS sorting to yield a population in which 77% of the cells were highly GFP – positive. PCR and Western blotting confirmed reductions of 40 and 42% in mRNA (**Figure 2A**) and protein (**Figure 2B**) levels in these cells, respectively, as compared to control, C6/LacZ^{GFP} cells. The reduction in IGF-IR expression levels resulted in a markedly decreased IGF-I responsiveness in these cells, as reflected in

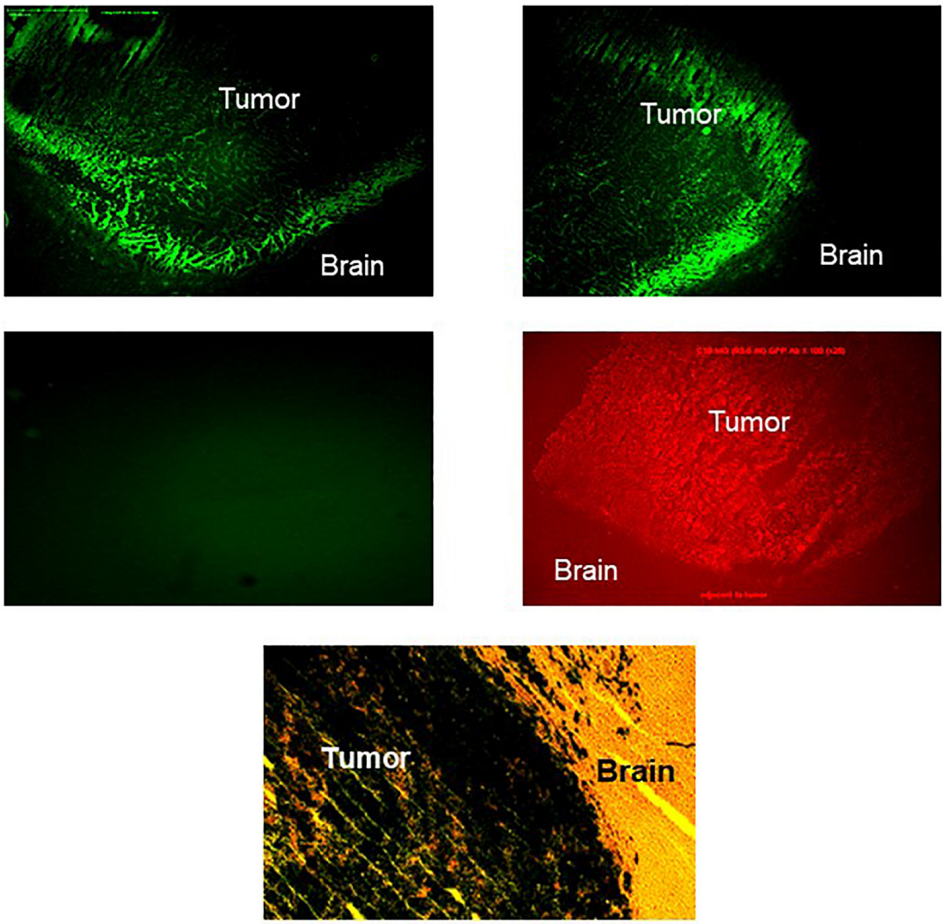

FIGURE 1 | Increased IGF-IR expression in the invasive margins of intra-cerebral rat glioma implants. Brain sections were prepared 20 days after the intra-cranial implantation of 10^4 C6 cells. The tissue was processed and immunostained as described in the section "Materials and Methods," using anti IGF-IR (**top panel, left** and **right**, showing two different areas of the tumor-brain margin) or anti-GFP **(middle panel, right)** antibodies. Control sections were incubated with goat anti rabbit IgG antibody only **(middle panel, left)**. The green emission background of the GFP positive glioma cells in all sections was minimized by lowering the exposure time (see **middle panel, left**). A parallel section stained for β-galactosidase activity and counter stained with hematoxylin and eosin is shown on the bottom (×120 magnification).

reduced cell proliferation revealed by the MTT assay (up to 53% reduction, **Figure 2C**), increased apoptosis in serum depleted medium supplemented with IGF-I, as measured by PI staining (2.2-fold increase in PI positive cells, **Figure 2D**), and reduced anchorage-independent growth, as assessed in a 3D spheroid assay (**Figure 2E**).

The reduction in IGF-IR-mediated functions had a marked effect on glioma cell tumorigenicity in the brain, following intra-cerebral implantation. While 80% of the rats injected with C6/LacZ^{GFP} cells were moribund by 24 days following intra-cerebral tumor implantation, all the rats in the C6/LacZ^{AS}-injected group were still alive at that time. Tumor volume measurements revealed that although C6/LacZ^{AS} cells could initially form small tumors, their ability to expand in the brain and produce large tumor masses was significantly impaired ($p < 0.0007$) relative to the controls (**Figure 3A**). A TUNEL assay performed on brain sections showed the presence of numerous apoptotic cells in the C6/LacZ^{AS}-injected rats as compared to no or few TUNEL-positive cells in the controls (**Figure 3B**), confirming that reduced tumor growth in the brain resulted from increased apoptosis in these cells. No significant differences in tumor volumes or the proportions of TUNEL-positive cells were observed when C6/LacZ and C6/LacZ^{GFP} tumors were compared. The profound effect that IGF-IR downregulation had on the growth of these cells *in vivo* was akin to the significant growth reduction observed in the spheroid assay (**Figure 2E**), consistent with results reported by others on the importance of IGF-IR for anchorage independent 3D tumor growth (20, 21).

When the effect of reduced IGF-R expression in the tumor cells on rat survival was subsequently analyzed, we found a significant increase in long-term survival of rats inoculated with C6/LacZ^{AS}, as compared to controls. Namely, while 86% of the rats injected with C6/LacZ^{GFP} cells were moribund by day 24 following tumor cell inoculation, 72% of the rats injected with C6/LacZ^{AS} cells were clinically disease-free until day 182, at which time they were euthanized ($p < 0.01$, **Figure 3C**).

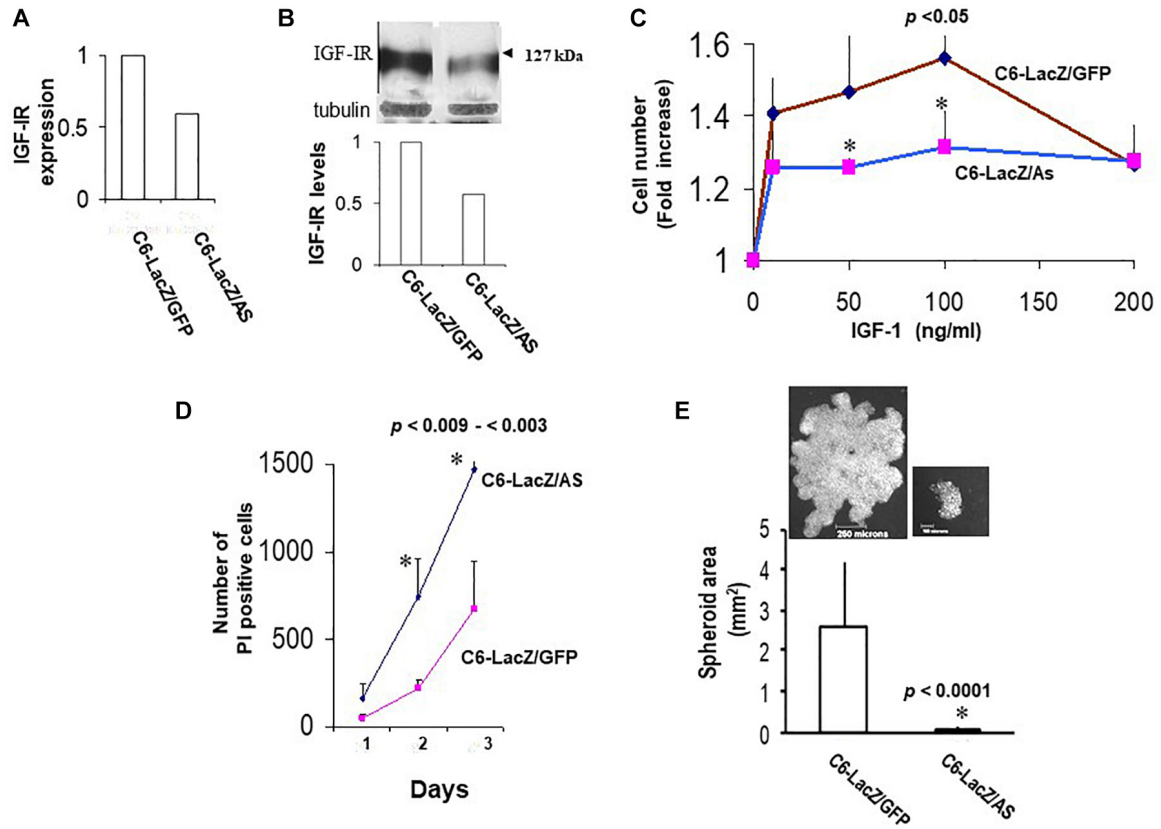

FIGURE 2 | Reduced IGF-IR expression in virally transduced C6 cells affects multiple IGF-I – regulated functions. IGF-IR mRNA expression levels **(A)** were measured by RT-PCR. Representative results of densitometry analysis (n = 2) shown in the bar graph are expressed as the ratios of the densities of IGF-IR:L19 bands relative to control C6/LacZ^{GFP} cells that were assigned a value of 1. Protein levels **(B)** were analyzed by Western blotting. Representative results of laser densitometry, shown in the bar graph (n = 2) are expressed as the ratios of IGF-IR:tubulin bands relative to C6/LacZ^{GFP} cells that were assigned a value of 1. Cell proliferation **(C)** was measured by the MTT assay. Shown are the results of a representative experiment of 3 performed. Data are expressed as the means and SD of triplicate samples (*p < 0.05 for 50 and 100 ng/ml IGF-I). Cell survival in IGF-I containing medium **(D)** was analyzed by PI staining. Glioma cells were plated in 4-chamber slides, first in complete medium and then in serum-free RPMI medium containing 100 ng/ml rhIGF-I. PI (1 mg/ml) was added to the chambers for 5 min at 37°C, on days 1–3 and the PI-permissible (dead) cells enumerated using an inverted microscope. Data are expressed as means (±SD) of triplicate samples (*p < 0.003 and p < 0.009 for days 2 and 3, respectively). Shown in **(E)** are means (±SD) of total spheroid surface area for the indicated cells. Images of representative spheroids for each of the cells are shown on top.

Solitary, Growth Arrested C6/LacZ^{AS} Cells Can Be Detected in the Brains of C6/LacZ^{AS} Injected Rats Several Months After Tumor Implantation

In brain sections prepared from clinically disease-free C6/LacZ^{AS}-injected rats, 182 days after tumor injection, we identified single LacZ positive cells in ipsilateral and/or contralateral regions of the brain (**Figure 4A**). In some animals, they were also seen around the choroid plexus in the ventricles (**Figure 4A**, bottom). These cells could also be detected by immunofluorescence staining using an FITC-conjugated rabbit anti β-galactosidase antibody (**Figure 4B**). Cells in all regions were solitary and no foci could be seen in a total of 150 sections (15 sections/rat, n = 10) examined. The robust expression of β-galactosidase indicated that the glioma cells were alive until the end of the experiment. However, no Ki-67 staining could be detected in these cells, indicating that they were in cell cycle arrest (**Figure 4B**). Two of the 10 rats injected with the C6/LacZ^{GFP} cells also survived for 6 months. We therefore used them to compare the incidence of growth-arrested solitary tumor cells in the two groups. Analysis of three sections/rat derived from different regions of the brain revealed that in animals injected with C6/LacZ^{AS} cells, the number of solitary LacZ^{+} cells was 20-fold greater than in C6/LacZ^{GFP} injected rats (**Figure 4C**). This suggested that the acquisition of a prolonged state of cell cycle arrest by C6/LacZ^{AS} cells was not random but rather a specific outcome of reduced IGF-IR expression in these cells.

Direct Intra-Cerebral Inoculation of vLTR-IGF-IR^{AS} Particles Also Inhibits Tumor Growth

In order to determine the ability of vLTR-IGF-IR^{AS} to target pre-established tumors growing in the brain, rats were injected

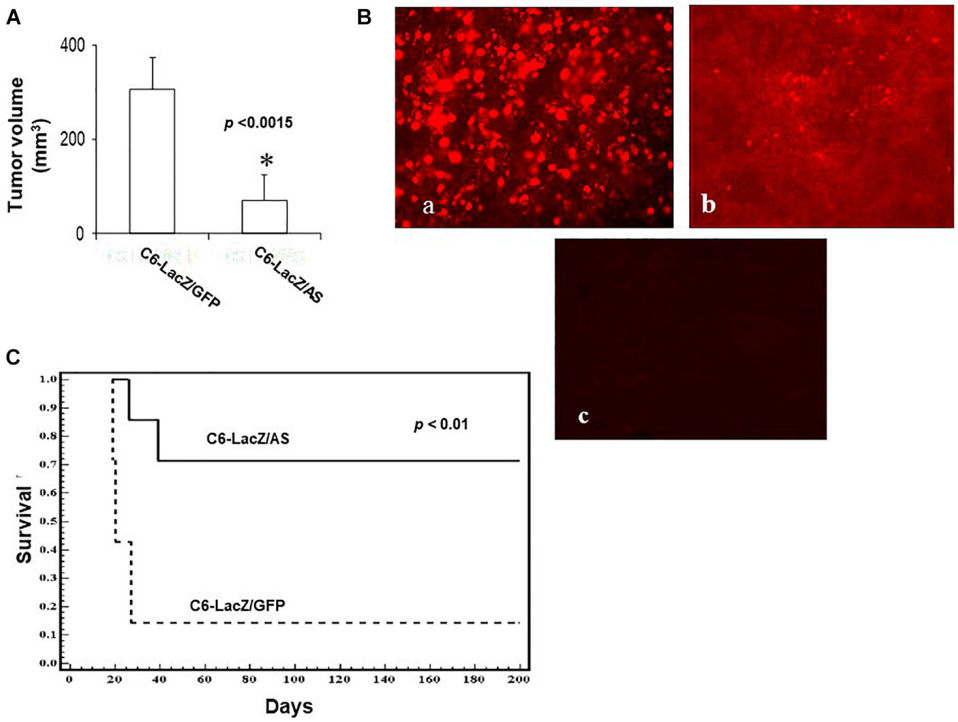

FIGURE 3 | Reduced intracerebral growth of C6/LacZ^{AS} cells. Rats were implanted with 10^4 cells intra-cranially and the brains removed for tumor volume measurement as described in section "Materials and Methods." Shown in **(A)** are the results of a representative experiment of two performed ($n = 5$ in each, $^*p < 0.0015$). Shown in **(B)** are results of a TUNEL assay performed on representative sections. Apoptotic cells were visualized using Cy3-conjugated streptavidin (**a**-C6/LacZ^{AS}; **b**-C6/LacZ^{GFP}). Control sections **(c)** prepared from C6/LacZ^{AS} injected animals were treated in the same manner but without the terminal deoxynucleotidyl transferase (TdT) enzyme. Long-term survival of rats implanted intra-cranially with 10^4 C6/LacZ glioma cells **(C)** was analyzed in three independent experiments using 9–14 animals per group. The animals were followed until the onset of morbidity, at which point they were euthanized. Disease-free rats were euthanized 182 days post tumor implantation ($^*p < 0.01$).

intra-cranially with 2.7×10^6 vLTR-IGF-IR^{AS} or vLTR-GFP particles, 6 and 13 days following the orthotopic implantation of the C6/LacZ cells. Fluorescence microscopy confirmed the presence of GFP$^+$ C6/LacZ cells, localized mainly at the advancing edge of the tumors (**Figure 5A**), indicating that the retroviral particles were able to transduce cells within pre-established C6/LacZ brain tumors. All the animals were euthanized 24 days post tumor implantation and tumor volumes were calculated. We found a 22% reduction in the mean tumor volume in rats inoculated with vLTR-IGF-IR^{AS} as compared to controls injected with the vLTR-GFP particles ($p < 0.026$, **Figure 5B**). Importantly, while 40% of the control rats were already moribund at this time, no morbidity was observed in the vLTR-IGF-IR^{AS} – injected group.

Human U87 Glioma Cells Transduced With vLTR-IGF-IR^{AS} Particles Also Have Reduced Growth *in vivo*

We subsequently investigated the effect of retrovirally induced suppression of IGF-IR expression on the growth of human glioma cells, using the U87MG cell line. Sublines U87MG-LacZ/AS and U87MG-LacZ/GFP were generated as described for C6 cells. The efficiency of gene transfer in these cells, as determined by flow cytometry was 48 and 94%, respectively. Western blotting revealed a 36% reduction in IGF-IR expression in U87MGLacZ/AS cells relative to controls (**Figure 6A**). This resulted in reduced IGF-IR signaling, as reflected in a 40% reduction in IGF-I induced ERK activation in these cells, as compared to control, U87MG-LacZ/GFP cells (**Figure 6B**).

The effects of IGF-IR suppression on the intra-cerebral growth of U87MG cells were more profound than those observed in the rat glioma model. In a tumor volume study (**Figure 6C**), all U87MG-LacZ/GFP–implanted, but none of the U87MG-LacZ/AS implanted mice had sizable tumors by day 35 post implantation. This was reflected in a significant difference in long-term survival because all mice implanted with U87MG-LacZ/GFP were euthanized for neurological symptoms by day 63 post implantation, while 70% of those implanted with U87MG-LacZ/AS cells were clinically disease free until day 176, at which time they were euthanized (**Figure 6D**). Interestingly, in nude mice implanted with U87MG-LacZ/AS cells, the presence of solitary β-gal$^+$ tumor cells in the brains was limited to the injection tracts (not shown) and could only be seen in mice sacrificed by day 35, when tumor volumes were measured, suggesting that reduced IGF-IR levels eventually caused the death of all U87MG cells.

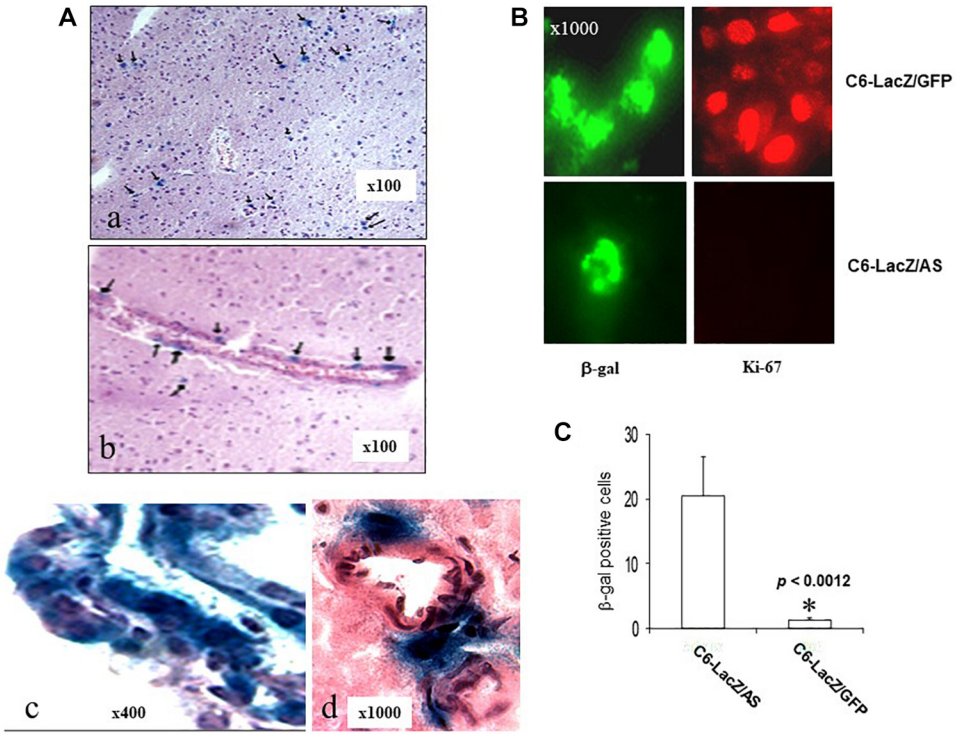

FIGURE 4 | Long-term survival of solitary, intra-cerebral tumor cells in C6/LacZ^{AS}-injected rats. Brain sections (10 μm) of rats that survived 182 days following C6-LacZ^{AS} implantation **(A)** were immunostained to detect β-galactosidase activity. Shown are solitary cells identified in the brain parenchyma **(a)**, adjacent to ventricles **(b)**, within the choroid plexus **(c)** and in the perivascular space associated with small arterial vessels **(d)**. Immunohistochemistry **(B)** was performed on sections derived from the indicated surviving rats using antibodies to Ki-67 (right) or β-galactosidase (left). LacZ positive cells in the sections were quantified **(C)** in five random fields (×100 magnification) per section and three sections were analyzed per brain. Shown are mean numbers of β-gal^{+} cells (±SD) in a total of 10–15 sections analyzed per group. *$p < 0.0012$.

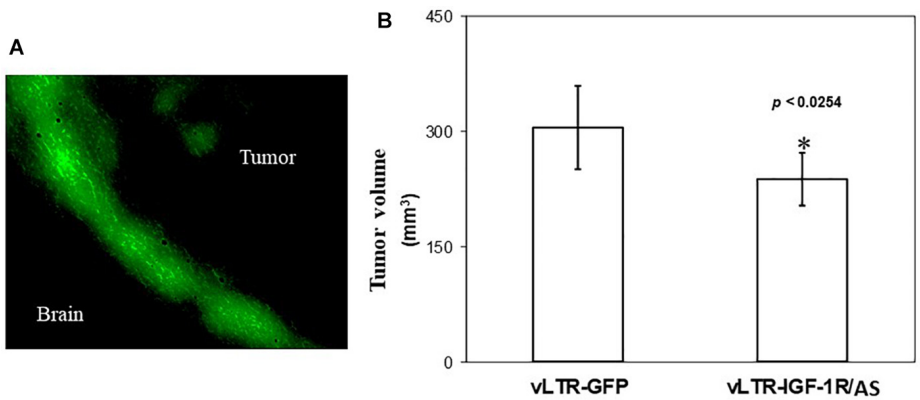

FIGURE 5 | *In vivo* targeting of pre-implanted rat glioma cells with IGF-IR antisense expressing viral particles reduces tumor expansion. Shown in **(A)** are intra-cerebral C6 cells following *in vivo* transduction with vLTR-GFP retroviral particles. GFP-expressing cells were observed mainly at the expanding edge of the tumors. Shown in **(B)** are mean tumor volumes (±SD) following two injections of vLTR-IGF-IR^{AS} or control vLTR-GFP particles, 6 and 13 days post orthotopic implantation of C6/LacZ cells ($n = 5$, *$p < 0.0254$).

DISCUSSION

The critical role that the IGF axis plays in the growth of GBM has been well documented in various experimental models (6, 22, 23). In early studies, the IGFs were shown to enhance 3D growth of glioblastoma (21) and more recently, high IGF-IR expression levels were identified as an independent prognostic factor associated with shorter survival, a poorer response to temozolomide (4) and resistance to anti-EGFR (24) and anti-PDGFR (25) treatments in GBM patients. Recently, it was shown

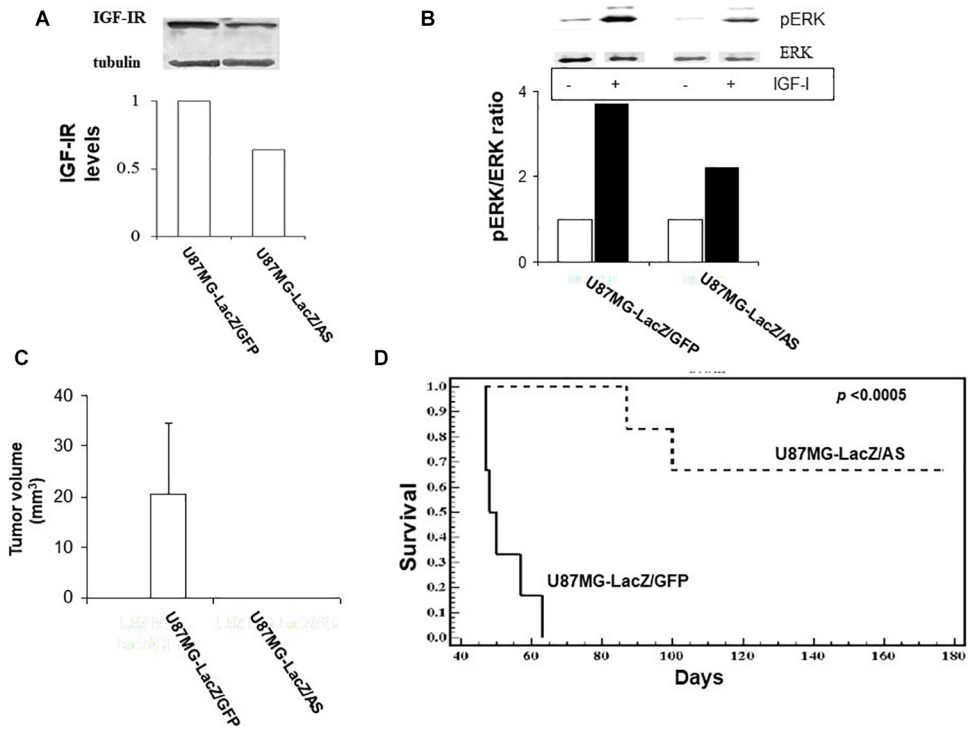

FIGURE 6 | Reduced IGF-IR expression in virally transduced U87MG cells inhibits IGF-I responsiveness *in vitro* and tumor growth *in vivo*. U87 cells were transduced with vLTR-IGF-IR^{AS} or vLTR-GFP particles. IGF-IR levels were analyzed by Western blotting **(A)**. Shown are the results of a representative blot ($n = 2$). Results of the laser densitometry, shown in the bar graph are expressed as the ratios of IGF-IR:tubulin relative to U87MG-LacZ/GFP cells which were assigned a value of 1. ERK phosphorylation **(B)** was measured following the incubation of serum-starved tumor cells with 100 ng/ml IGF-I for 10 min. Blots were probed consecutively with antibodies to phospho-p44/42 ERK and p44/42 ERK. Shown are the results of one representative experiment of two performed. Results in the bar graph are expressed as mean increase in pERK:ERK ratios relative to control, untreated cells (analyzed in the same gel) that were assigned a value of 1 ($n = 2$). Nude mice were injected intra-cranially with 10^5 U87 cells as indicated. Tumor volumes **(C)** were measured on day 35 post injection and survival **(D)** was analyzed in separate groups of mice injected as above. Shown in **(C,D)** are pooled data from two separate experiments, each performed with 6 nu/nu mice per group. $^*p < 0.0005$.

that elevated expression of IGF-IR and IGF-II in GBM were associated with poor patient survival and that paracrine IGF-IR/IGF-II signaling promoted the expansion of a chemoresistant glioma subpopulation (26). The levels and patterns of IGF expression were also shown to correlate with histopathologic grade in diffusely infiltrating astrocytomas (27).

In the brain, glioma cells can respond to IGF ligands originating from several potential cellular sources. They may produce the factor and utilize it in an autocrine fashion (26, 28), or they could utilize IGF-I produced by reactive astrocytes or microvascular cells exhibiting endothelial/pericytic hyperplasia at the margins of tumor infiltration, as was shown by Hirano et al. (27). Interestingly, while in normal cells, IGF-I typically binds to IGF-IR with (5–10-fold) higher affinity than IGF-II, glioma cells were shown to express a receptor with a unique IGF-IRα subunit of higher molecular size that has similar affinities for both ligands (29). This provides the tumor cells with a unique advantage, because they are able to respond more effectively to IGF-II that is produced at high concentrations in the brain (30, 31). This dual, high affinity binding may also limit the efficacy of therapeutic strategies designed to target individual ligands (32) and underlines the importance of targeting the receptor (9, 32) or both ligands. Targeting IGF-IR expression using strategies such as gene therapy also has the added advantage of reducing the levels of receptor available to translocate to the nucleus, where it was shown to play a regulatory role, independent of its membrane-bound functions (33).

The critical role of the IGF axis in glioma progression has been confirmed in multiple studies including by Resnikoff et al. (34) who used an antisense strategy to silence IGF-IR expression in C6 cells. However, few studies have actually examined the effects of reduced IGF-IR expression, or function, on glioma cell growth and spread orthotopically, in the brain (7, 35). Using the C6 cells, we document here high expression of IGF-IR at the invasive margins of expanding tumors, suggesting that glioma cells in the margins of expanding tumors are uniquely positioned to respond to IGF ligands produced by the microenvironment, an interaction also documented in a recent study by Quail et al. (36). Interestingly, high IGF-IR levels were also documented at the invasive margins of other tumors such as colorectal carcinoma liver metastases, where they were shown to coincide with high IGF-II expression in adjacent, hepatic stromal cells (37).

Viral particles have been employed by others to deliver antisense RNA into tumor cells. This approach was used to target oncogenes such as K-Ras (38) and c-fos (39, 40), growth and survival factors such as IGF-I (41), TGF-β (42) and bcl-2 (43)

and, more recently, telomerases (44). The present work adds to this body of evidence and shows that growth factor receptor-targeting by antisense RNA delivered via VSVG-modified viral particles can affect the tumorigenic phenotype of the cells when delivered intra-tumorally *in vivo*. The results also establish the feasibility of obtaining effective levels of suppression for the antisense-targeted gene, while maintaining high expression levels for a second, reporter gene such as EGFP coexpressed in a bi-cistronic transcript.

Our data show that a reduction in IGF-IR expression could affect intra-cerebral glioma growth in one of several ways. While the majority of C6/LacZ^{AS} cells became apoptotic by day 24 following injection, others survived, and either continued to proliferate, eventually leading to morbidity, or they entered a prolonged state of growth arrest as solitary cells. While the mechanisms determining ultimate tumor cell fate in our model remain to be fully elucidated, several possibilities can be proposed. The C6/LacZ^{AS} and U87MG-LacZ/AS cells used in this study were polyclonal, consisting of cells with variable IGF-IR expression levels that could affect the susceptibility to, and outcome of antisense-mediated suppression. Moreover, the transgene expression levels were also variable as reflected in a range of GFP levels in these cells. Although the use of a heterogeneous population can potentially lead to the range of responses that we observed, it was our preferred approach because it more closely mimics the potential effects of direct viral transduction in a clinical setting. In this context, it is relevant that a threshold effect was previously documented for IGF-IR-mediated signaling and function. Namely, in embryonic fibroblasts derived from IGF-IR - null mice ectopically expressing IGF-IR, a requirement for a threshold of 1.5×10^4 receptors per cell was demonstrated for IGF-I-induced DNA synthesis and of 2.2×10^4 and 3×10^4 ligand-binding sites per cell, respectively, for cell proliferation and transformation (45, 46). Similar observations were reported in a fibrosarcoma model (47) and in our own studies with a Lewis lung carcinoma model (48). Thus, C6 cells may have become apoptotic if their IGF-IR expression levels were below the threshold required for survival, or entered cell cycle arrest if they expressed the requisite receptor levels for survival but not for cell cycle entry. A variable response of the tumor microenvironment to tumor cells with different IGF-IR expression levels may also have contributed to the divergent outcomes, as IGF-IR signaling levels were shown to affect tumor cell immunogenicity (23, 34).

The latter finding was, in fact, the basis of a small clinical trial where immunization of glioma patients with autologous glioma cells expressing antisense IGF-IR had a beneficial effect, resulting in clinical and radiographic improvements in 8 of 12 patients, including three spontaneous or postsurgical regressions at either the primary or a distant intracranial site (49). Exosomes released by immunizing glioma cells were subsequently implicated in the immunizing effect. While we cannot at present rule out a contribution by the innate and/or adaptive immune response to the tumor inhibitory effect of IGF-IR silencing observed in our study, our finding of profound growth suppression of U87MG cells that were orthotopically implanted in nude mice suggest that it was not predicated on an intact T cell immune response. Of interest in this context is a recent study by Quail et al. (36), who found that in a transgenic mouse model of spontaneous GBM, cells that were dormant following colony stimulating factor receptor 1 (CSF-1R) inhibition became resistant to the treatment and were rescued from the dormant state by stroma-derived IGF-I, adding support for the role of IGF signaling in maintaining the balance between tumor cell proliferation, death and the acquisition of a dormant state. The reason for the prolonged survival of a small but detectable number of control C6/LacZ^{GFP} cells in a small number of control rats is presently unclear and may also be related to clonal heterogeneity in this tumor line.

The C6 cells are chemically induced rat brain cancer cells widely used as a glioma model for *in vitro* and *in vivo* studies (50). In recent years, the relevance of these cells as a model for human glioma has been questioned because their origin as chemically induced brain tumors may better represent "gliosarcomas" (51). It should be noted however, that in the present study, and in line with other studies (52), we did not observe the sarcomatous differentiation characteristic of gliosarcomas. Furthermore, in different studies, both the phenotype and immune microenvironment of experimental rat C6 gliomas were shown to resemble those of human glioblastoma (53, 54). Also of note, the clinical features and outcomes of gliosarcoma and glioblastoma, as revealed in clinical studies are essentially indistinguishable (55). Taken together with the results we obtained with the U87MG model, we believe that the present findings are of relevance to the management of human glioma. Of interest, loss of PTEN was identified as a resistance mechanism against IGF-targeting drugs in high grade gliomas (56) and other malignancies (57). Intriguingly, our results have shown that IGF-IR silencing was effective in PTEN-null U87MG cells (58). While the mechanism underlying the heightened sensitivity of these cells within the brain microenvironment remains to be elucidated, it is possible that the MEK/ERK pathway that is activated by IGF-I in these cells (as shown in **Figure 6**) determines their sensitivity to IGF-IR inhibition, in the presence of aberrant PI3K/PTEN/Akt signaling.

Type 1 insulin-like growth factor targeting may be highly beneficial in combination therapy with other anti-cancer drugs, because IGF-IR signaling was identified as a resistance mechanism for both chemo and targeted therapies (2). In particular, resistance to EGFR inhibition in human glioma cells was attributed to compensatory signaling by IGF-IR (59), and insulin receptor/IGF-IR signaling was shown to confer resistance to Gefitinib in EGFR-dependent glioblastoma, through compensatory AKT activation (24). Co-targeting of the IGF-IR and EGFR axes may therefore provide an efficacious therapeutic strategy for GBM (24). It may also be relevant to brain metastases originating from other malignancies, such as breast cancer (60).

CONCLUSION

In conclusion, our data demonstrate that a gene therapy approach to IGF-IR silencing *in situ* holds promise as a strategy for limiting glioma growth *in vivo* either as monotherapy or in combination with other drugs. However, they also raise a cautionary note that

a better understanding of the role of the IGF-IR in regulating the delicate balance between glioma cell death, dormancy and proliferation will be critical to the optimization of this approach and to effective control of residual disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the McGill University Animal Care Committee.

AUTHOR CONTRIBUTIONS

AAS planned, performed, and analyzed all the experiments and drafted the manuscript. JN provided resources and guidance for the performance of animal experiments. PB oversaw the study, planned the experiments, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Technical Advancements for Studying Immune Regulation of Disseminated Dormant Cancer Cells

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Metastases are a major cause of cancer-related death and despite the fact that they have been focus of intense research over the last two decades, effective therapies for patients with distant secondary lesions are still very limited. In addition, in some tumor types metastases can grow years after the patients have been declared clinically cured, indicating that disseminated cancer cells (DCCs) persist undetected for years, even decades in a quiescent state. Clinical and experimental data highlight the importance of the immune system in shaping the fitness and behaviour of DCCs. Here, we review mechanisms of survival, quiescence and outgrowth of DCCs with a special focus on immune-regulation and we highlight the latest cutting-edge techniques for modelling the biology of DCCs *in vitro* and for studying the metastatic niche *in vivo*. We believe that a wide dissemination of those techniques will boost scientific findings towards new therapies to defeat metastatic relapses in cancer patients.

Keywords: dormancy, metastasis, tumor microenviroment (TME), immune cells, labeling techniques

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CLINICAL PROBLEM

According to a recent analysis, the proportion of cancer deaths with metastases as contributing cause, ranged from 9.3% for CNS cancers to 90.4% and 80.2% for ovarian and colon cancer, respectively (1, 2). Metastases can be detected in concomitance with the primary tumor (synchronous) or at a later stage (metachronous). Although most tumors cover the same steps of metastatic dissemination (i.e., extravasation, dissemination through blood or lymphatics, intravazation, and establishment in the metastatic niche), the time required to form overt lesions significantly differs according to the tissue of origin and cancer subtypes. While breast, prostate, renal cell cancers, as well as sarcomas and melanomas show long latency and the time required to develop metachronous metastasis might reach 15 years, 85% of relapses from colon cancer are detected within 3 years (medium latency), and lung cancers often spread at distant sites within a few weeks (short latency) (1, 3–5). When the time required for a DCC to form an overt metastasis after the removal of the primary tumor is long (arbitrarily usually set as 5 years), latency is often referred to as "dormancy". Importantly, different metastatic latencies might underlie different mechanisms in the acquisition of aggressive traits, and at the same time significantly impact on our capacity to intervene, as the time preceding the metastatic onset offers a therapeutic window so far underexploited. Thus, it is a priority to understand the biology of DCCs, cell intrinsic and extrinsic determinants of their death, survival and growth at the secondary site.

One factor that profoundly affects relapse of DCCs is the cell of origin and its genetic landscape, as exemplified by breast cancers. More than half of breast tumors positive for estrogen receptor (ER) relapse after 5 years of diagnosis and mastectomy, with a progressive increase in recurrence risk from 5 to 20 years in patients treated with adjuvant endocrine therapy (6, 7). This is in contrast with data from patients with ER negative breast cancers, where relapses mostly occur within the first two years (7). Interestingly, while averaging over a long time is required for meta-analysis of different case series, analysing events at shorter intervals in homogeneous case series allows the identification of a multi-peaks pattern of breast cancer recurrence (8, 9). This observation leads to a fundamental question: why do dormant DCCs (DDCCs) reawaken in cured patients with no apparent clinical condition? Beyond stochastic local perturbations, paraphysiological signals involved in exit from dormancy have yet to be identified, but candidates are, for example, hormones or factors related to lifestyle, such as diet (10, 11).

Interestingly, a recent report provided experimental evidence in support of a longstanding clinical observation, i.e., the effect of systemic inflammation on reawakening of DDCCs (8, 12). The paramount role of inflammation and immune surveillance on the behaviour of DDCCs has been unequivocally demonstrated by inadvertently transplanted malignant tumors (13–16). Demand for transplant organs far exceeds available donors, thus, occasionally, donors with a history of cancer were accepted provided that they were disease-free long enough to be considered cured (>10 years). In several cases, recipient patients developed metastases after transplantation of heart, kidney, lungs or liver. Most frequently transplanted tumors were renal cell cancer, cutaneous malignant melanoma, malignant glioblastoma (which is usually considered non-metastatic). Several concepts can be drawn from these reports: i) the presence of a malignant cancer was unknown for some donors, supporting the concept of an early dissemination, ii) the presence of DDCCs in organs that are not considered sites of secondary tumors, indicating that dissemination is not a prerogative of few organs, iii) immune system has a central role in controlling outgrowth of DDCCs, as when the organ was removed and immunosuppression discontinued, malignant cells were rejected by the host (host versus graft). This is supported by the empirical evidence that more metastatic lesions are observed in immunocompromized experimental mouse models compared to wild-type strains (12). The importance of the immune control of DDCCs is further reinforced by the clinical evidence showing discrete peaks of recurrence in patients after resection of the primary tumor, likely as a consequence of systemic inflammation (2, 12, 17). Importantly, perioperative resolution of the inflammatory status prevents outgrowth of otherwise DDCCs (12, 17).

These clinical evidences highlight the role of immunity in the control of DCCs survival and growth and strongly support a better understanding of the dynamic and complex immune tumor microenvironment (TME) at the metastatic site at a single cell level. However, this has been difficult to achieve so far due to the lack of tools to study local interactions between DCCs and their neighbouring cells. In this review we will first clarify key definitions in the dormancy phenotype and then summarise the current knowledge on the non-immune as well as immune-related mechanisms of dormancy. At the end of the review we will highlight recent technological advances that might greatly push forward our knowledge of the molecular mechanisms associated with dormancy.

DEFINITIONS

Dormancy can be used to describe two very distinct phenomena: primary tumor dormancy and metastatic dormancy. The former indicates the time required by an evolving cancer cell to overcome oncogene-induced senescence or apoptosis, metabolic adaptation, evade immune clearance and induce neoangiogenesis, thus forming a detectable tumor mass (18). Metastatic dormancy, instead, indicates the time required by a DCC to overtake the attrition due to seeding in a hostile environment and develop an overt lesion. Although in some cases, determinants of dormancy might be shared among primary tumor and metastases [such as ERK/p38 ratio and fibronectin (19, 20)], they are likely to be distinct processes.

Another distinction often used is between "cellular dormancy", i.e., cells undergoing reversible G0/G1 cell cycle arrest, and "tumor mass dormancy", indicating small clusters of cells where proliferation is balanced by death induced by lack of nutrients (angiogenic dormancy) and/or by immune clearance (immune dormancy). Although useful to rationalize the dormant phenotype, this sharp distinction has little experimental support and likely the two conditions coexist, with DDCCs dynamically fluctuating between the two states during their history. For example, a small, but detectable, proportion of early DCCs (eDCCs) from experimental breast cancers are found positive for proliferation markers (21), despite they are often referred to as "non-proliferative". Moreover, Aguirre-Ghiso and colleagues showed, with an elegant genetically-encoded fluorescent marker dilution assay, that post-hypoxic DCCs, which are much less proliferative than post-normoxic DCCs, still undergo considerable proliferation over two weeks (22). This dynamic heterogeneity is not unique to DDCCs, as a significant number of Ki67-negative cells are found even in DCCs from aggressive cell lines, such as MDA-MB-231 (23). The development of longitudinal assays that keep track of the proliferative history of DCCs will help to understand if cellular and tumor mass dormancy are static or dynamic entities and which of them contributes to aggressive lesions.

MECHANISMS OF SURVIVAL, QUIESCENCE, AND REAWAKENING OF DDCCS

The fate of disseminated cells is driven by a combination of cell intrinsic, extrinsic and stochastic events (1). Cell intrinsic

programs involve oncogenes and tumor suppressors, membrane proteins (integrins, receptors etc…), intracellular components (such as cytoskeletal proteins and mechanotransducers), signaling pathways and sensors that integrate genetic and microenvironmental inputs and translate them into cellular processes. Cell extrinsic programs include triggers from the niche, such as stromal cells, tissue architecture, biophysical and biochemical cues, as reviewed in (24, 25). Intrinsic and extrinsic signals do not act on their own, rather they are nodes and connectors of a complex and dynamic network where extrinsic signals from TME (organ specific or shared) funnel into key intrinsic signaling hubs.

Immune-Independent Mechanisms

P-ERK/P-p38 ratio is perhaps the most widely validated dormancy hub so far (25, 26). While activated ERK drives exit from dormancy and growth, P-p38 promotes growth arrest *via* several mechanisms, such as MSK1, DEC2, NDRG1, NR2F1, and ultimately p21 and p27 induction (26, 27). Several signals converge on p38, such as TGFβ2 (28), BMP7 (29) as well as the metastasis suppressors MKK4 and Nm23 (30–32). Another determinant of dormancy/growth signaling is the PI3K/Akt/mTOR axis, whose activation drives survival and exit from dormancy (33–37). Different integrin dimers, often in conjunction with Src, have been consistently linked with survival of DDCCs and/or metastatic outbreak (20, 24, 38–45). Several signaling pathways have also been linked so far with quiescence and metastatic fitness: TGFβ and BMP pathways (23, 28, 29, 46–48), canonical and non-canonical Wnt pathway (21, 49–51), YAP/TAZ (41, 42), Notch (49, 52), JAK/STAT (53, 54). Recently, ER stress response and autophagy have been convincingly linked with survival of DDCCs *in vitro* and *in vivo* (55–58).

Several fibrous and non-fibrous ECM proteins have been shown to be key determinants of metastatic fitness (40): collagen I (39, 59), fibronectin (20, 38, 60), periostin (23, 50), tenascin C (49), thrombospondin (23, 41). Beside ECM proteins, hypoxia present at the primary site primes breast cancer DCCs for dormancy upon seeding to secondary organs (22).

Stromal cells provide organ-specific niches that regulate both quiescence and reactivation. Bone is probably the most characterised niche, since it is the preferred metastatic target of prostate and breast cancer (61). Osteoblasts release the growth arrest specific 6 (GAS6) ligand that binds to the Axl subfamily of receptors inducing dormancy (47, 62–65). Importantly, DDCCs can hijack endogenous signals regulating hematopoietic stem cells' (HSC) reversible quiescence. For example, the chemokine CXCL12 from bone endothelial cells and mesenchymal progenitors induces dormancy in DCCs and HSC (1, 66–69). On the contrary, in preclinical models of bone metastasis, RANK-stimulated osteoclasts are reported to mobilize DDCCs and trigger proliferation (43, 70–72). Lung is another common homing site for DDCCs and interaction of breast DDCCs with type I pneumocytes is key for the activation of a dormant gene program in DCCs (20). In this context, cellular protrusions are required to gather survival signals from the microenvironment (20, 73, 74). Importantly, stromal derived BMP2 and TGFβ2 keep DDCCs in a latent state (28, 46), while collagen-rich fibrotic lung transforms DDCCs into aggressive metastatic cells (39), a similar mechanism was observed in fibrotic liver (75). Lastly, the perivascular niche regulates DCCs behaviour and chemoresistance in multiple organs (23, 45, 76).

Immune-Related Mechanisms

Immune cells are known to play a key role in shaping the TME in primary tumor and metastasis (77–79) and several evidences show that their recruitment at distant sites anticipates cancer cells colonization (59, 80–86). Moreover, extracellular vesicles (EVs) from the primary tumor have been shown to induce a premetastatic niche at the metastatic site [reviewed in (87, 88)]. Notably, the protein content of exosomes is critical to their function and it defines where cancer cells metastasise (89) and also influences response to chemotherapy of DCCs (90).

Because of their acknowledged tumor modulatory function immune cells have not surprisingly become a valid therapeutic target. Immunotherapy has finally proven its efficacy in treating patients and promises to further change the standard of care for cancer treatment in the coming years (91–94). However, a complete resolution of the TME as well as the understanding of this local crosstalk is far to be achieved, possibly limiting the efficacy of current immunotherapeutic options to a small number of patients. This local crosstalk has been shown to also occur *via* EVs. Immune cell derived exosomes have been initially shown to function as immunomodulators by carrying molecules able to induce a T-cell response (95, 96). However, metastatic cells can release exosomes expressing PD-L1 on their surface and are therefore able to suppress cytotoxic T cells (97–99). These mechanisms might influence a positive response to immunotherapy. Moreover, a further boost in immunotherapy might come from a better understanding of the immune diversity in the TME and the way immune cells locally interact, as this can help to predict therapeutic responsiveness (91, 100). Since immune cells are important in limiting metastatic outgrowth and keeping DCCs in an indolent state (5, 26, 101, 102), it is tempting to foresee a role of immunotherapy in targeting dormant DCCs (103). However, this possibility is currently restrained by a limited understanding of how immune cells interact with DCCs. In the next sections we will summarise the current knowledge on the role of immune cells specifically in metastatic dormancy.

Innate Immunity and Dormancy

Macrophages have long been known to play a role in cancer (77) and the intriguing finding that they polarise their status to support cancer growth paved the way for studies on immune cell pro-tumorigenic functions. For example, macrophages support tumor growth by several means, among them a direct inhibition of tumor suppressive immune cells (104, 105). Macrophages are able to directly promote breast DCCs survival in the lung *via* a VCAM1-α4 integrin binding (44). Interestingly, the aberrant expression of VCAM1 in bone-disseminated breast DCCs promotes the recruitment of monocytic osteocytic progenitors and subsequent transition from indolent growth to overt bone metastasis (43).

Macrophages also sustain early dissemination and metastasis in the HER2+ model of breast cancer (106) and have been shown to interact with residual tumor cells and promote tumor recurrence in a HER2-driven breast cancer (107).

Neutrophils represent another abundant component of innate immunity whose contribution in cancer has only started to be elucidated in the last few years (108). Neutrophils have been shown to boost lung metastasis from breast cancer (81, 109–111) and to reawaken DDCCs (41, 107). Their ability to reawaken DDCCs in the lung following LPS exposure is strongly dependent on the release of neutrophils extracellular traps (NETs) (41). Notably, the metastatic outgrowth of DDCCs induced by LPS-mediated inflammation is rescued following neutrophil depletion, but not when depleting macrophages with anti-CCL2 (107), indicating a unique role for neutrophils in this context.

Another innate immune population, the NK cells, has been associated with the clearance of DCCs. NK cells play a key role in immune surveillance during metastatic dissemination (112). Indeed, the expression of NK cell-activating ligands on cancer cells is critical for their clearance (113) and the upregulation of NK cell-activating receptors render cancer cells more susceptible to NK cell-mediated killing (114). Moreover, neutrophils-mediated NK-cell depletion promotes outgrowth of disseminated carcinoma cells (115). By using a "latency competent model" of breast and lung carcinoma, Massague and colleagues showed that while NK cells clear most of the disseminated cells at first instance, some cancer cells stochastically enter quiescence and downregulate ligands for NK cells to evade the immune surveillance. Importantly, these quiescent DCCs keep their tumorigenic potential and can re-enter cell cycle to metastases when NK surveillance is released (116).

Adaptive Immune System and Dormancy

Cancer immunoediting has been recognized as a process by which the immune system controls cancer growth, with a primary role of adaptive immunity (117, 118). Schreiber and Smyth laboratories made important contributions to show how T cells maintain cancer cell quiescence and how the depletion of CD4+ and CD8+ cells, but not NK cells, allows primary tumor growth in a carcinogen-induced model of sarcoma (119, 120). A key role for a subpopulation of T cells, the tissue-resident memory CD8+ cells, in maintaining a durable immune-cancer equilibrium, has also been shown in skin melanoma (121).

Importantly, T-cells also control cancer outgrowth when DCCs colonize secondary sites. Persistent endoplasmic reticulum (ER) stress plays a role in maintaining pancreatic DCCs quiescence and protecting them from a CD8+ T-cell-mediated response. Indeed, the combination of ER stress relief and T-cell depletion allows liver metastasis formation (57). Another study supports a pre-eminent role for CD8+ T-cells, but not CD4+ cells, in the immunosurveillance of DCCs in a model of spontaneous melanoma (122). CD8+ T-cells have also been shown to induce a state of dormancy in murine B cell lymphoma *via* the production of INFy (123), while in fibrosarcoma, either CD8+ T-cell or NK cell depletion lead to spontaneous metastasis in immune-competent mice. An orchestrated response involving different immune subpopulations has been suggested in a model of chemotherapy-induced dormancy in ER negative breast cancer. Here, the signaling activation of the IRF7/IFN-β/IFNAR axis following chemotherapy is associated with a reduction in granulocytes and expansion of T and B lymphocytes and dendritic cells (124).

IN VIVO MOUSE MODELS TO STUDY IMMUNE-DDCCS CROSSTALK

In the past decades several mouse models have been generated, each of them with strength and limitations in modeling the metastatic cascade [reviewed in (125)]. Experimental metastatic spread can be achieved i) by spontaneous dissemination of cells after formation of a primary tumor (either by cancer cell transplant or genetically-induced) or ii) by injecting cancer cells in the bloodstream (either allografts of mouse cells, or xenografts of human cells). The former have the advantage to mimic all the stages of metastatic colonization, the latter are more rapid and allow genetic manipulation of the injected cells, a prerequisite for some labeling techniques described later. Transgenic mouse models of dormant/indolent metastatic mammary cancers are worth mentioning, because they provide a good opportunity to study DDCCs in an *in vivo* immunocompetent animal. So far, three transgenic breast cancer models with spreading of indolent BCCs (breast cancer cells) have been reported: MMTV-Her2 (126), MMTV-PyMT (127), MMTV-Wnt1 (128). MMTV-Her2 and MMTV-PyMT models were used to support the early dissemination hypothesis, whereby dormant BCCs could be retrieved from the lung before the detection of the primary tumor lesion (127). Similarly, mammary cancers originating from the MMTV-Wnt1 transgene spread asymptomatic cells to lung and lymph nodes (128). Importantly, disseminated cells can be reawakened from dormancy following systemic triggers like bone marrow transplant or surgery. More recently, MMTV-Her2 and MMTV-PyMT mice have been used to describe the role of progesterone receptor, Her2 and partial-EMT into early dissemination (21, 126). The main limitation of the aforementioned models is that murine and human immune systems have important differences that undermined clinical translation of several preclinical findings. Moreover, the use of human cancer cells requires the use of immunodecifient or immunocompromised mice, which obviously fail to capture DDCCs-immune TME crosstalk.

Development of "humanized mice" started thirty years ago with the aim of studying human diseases in mice with a human immune system. Humanized mice are immunocompromised mice transplanted with human peripheral blood mononuclear cells (PBMCs), hematopoietic stem cells (HSCs) or human fetal tissues (thymus and liver) (129). Engraftment of patient-derived xenograft, or PDX (tumor fragments or single-cell suspension from tumor resections), are also employed to reconstitute the TME in mouse models. So far these models have been exploited mainly as preclinical testing platforms, as treatment responses in PDX have been correlated to those observed in patients in several

cancer types (130), but they hold great potential to uncover previously overlooked human-specific aspects of immune-DDCCs crosstalk.

TECHNOLOGICAL ADVANCES IN STUDYING THE TUMOR MICROENVIRONMENT

Advances in biomaterial technologies, including 3D bioprinting, are fundamental to model TME *in vitro* (131–136). The use of complex multi-cultures to mimic and perturb metastatic dormancy *in vitro* has been rapidly expanding, as reviewed in (24). More recently, the advances in microfluidic technologies are also boosting the development of cancer-on-chip models to better recapitulate multiple parameters of the TME complexity *in vitro* (137, 138) (**Figure 1**).

Biomaterial Technologies and 3D Bioprinting

The availability of new biomaterials also improved the studies of metastatic and immune cell interactions *in vivo*. Advancements in biomaterials allow to mimic the natural architecture of human tissues with scaffolds of tunable properties, either of natural (for example Matrigel) or synthetic origin (such as PCL, PLGA or PEG, polycaprolactone, polylactic-co-glycolic acid, polyethylene glycol, respectively) (132, 134). 3D scaffolds have been used to study the effects of ECM components and physical tissue properties as well as to dissect interactions between disseminated cells and stromal cells, such as fibroblasts, endothelial cells and macrophages (24, 134). Heterotopically implanted 3D hydrogels have been used to recreate artificial metastatic niches *in vivo*. Interestingly, following implantation they were infiltrated by immune cells and able to attract DCCs (139–141). Moreover, these systems can be easily manipulated to release cytokines and attract specific immune populations (140), representing a powerful tool to study how metastatic cells interact with host cells *in vivo* at a molecular level. However, they do not reflect the actual metastatic site composition whose replication remains difficult. This challenge has been partially overcome for leukemic tumors, thanks to the ability of HSC and progenitor cells to engraft and re-create a bone marrow environment. Humanized bone marrow environments have been used to study cellular interactions with human HSCs as well as malignant leukemic cells (142, 143). Interestingly, the engineered human bone-marrow niche can recapitulate main features of the pre-metastatic niche and attract DCCs, allowing to study the progression of the metastatic cascade (144). Importantly, 3D scaffolds have been successfully used as platforms for drug screening.

Three-dimensional bioprinting represents the most sophisticated strategy to achieve spatial control of matrix properties, integration of perfusable vascular networks and precise cancer-stroma cellular interaction (135). With 3D bioprinting, tissue spheroids, microcarriers, cell clusters, pellets, biomaterials and/or decellularized ECM can be deposited as bioinks under the control of computer designed patterns (135). With this technology, cancer models for several tissues have been generated, suggesting that metastatic niches as well could be designed in the near future.

Decellularized Organs and Precision Cut Tissue Slices

Hundreds of ECM proteins and carbohydrates are known to date, and their combination is key to the specificity of any cellular niche. Thus, reconstituting the exact ECM composition *in vitro* is almost impossible. For these reasons, several groups developed protocols to remove the cellular components of cell/ECM constructs, leaving decellularized ECM (dECM) that can be used for advanced *in vitro* model systems. dECM can be derived from native tissues or from tissues/organs generated *in vitro*. Decellularization protocols include chemical, physical or enzymatical approaches (or combinations) and the method

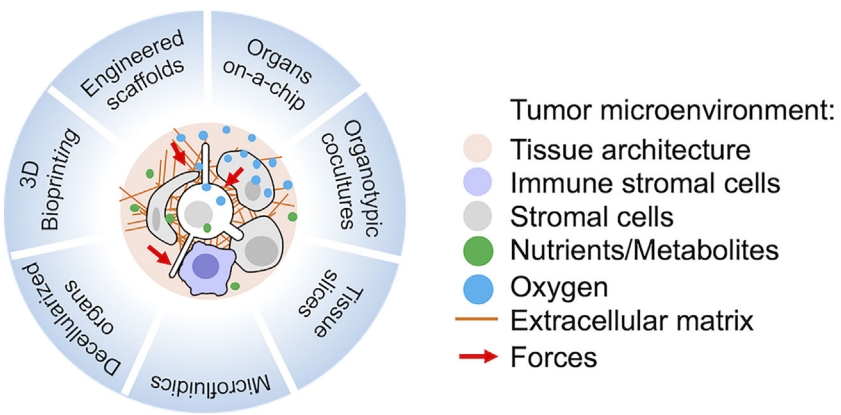

FIGURE 1 | Recent technologies suitable for the study of metastatic tumor microenvironment (TME) *in vitro* or *ex vivo*. Relevant reviews with references to the original works and protocols are provided in the main text.

clearly affects how much the resulting dECM resembles the native ECM of origin (145, 146). Decellularized tissues have been used to mimic breast cancer colonization of lungs (147) and adipose tissue (148). Importantly, dECM can be derived from patients, this allowed Pinto and colleagues to study crosstalk between colon cancer cells and macrophages within dECM from healthy and compromised tissues (149).

Long term *ex vivo* cultures of precision cut tissue slices and decellularized organs could be repurposed to study TME-DDCCs crosstalk in several secondary organs, such as lung, liver and brain (146, 150–153). Moreover, the use of intravital imaging technology combined with skin-fold chambers or optical windows has also allowed researchers to examine complex events *in vivo*, particularly in the context of primary tumors (154–156), but also in studying metastasis in organs such as bone, brain, liver and lung (157–159). However, the study of the metastatic TME *in vivo* remains technically difficult, particularly at an early stage of the disease, when small tumor nodules need to be spatially located in the metastatic tissue. This challenge is even bigger when dormant cell clusters or single DCCs need to be visualised and their neighbouring cells identified.

Microfluidic Systems and Organs-on-a-Chip

Another significant technological improvement for the design of metastatic niche *in vitro* is the development of microfluidic scaffolds. These platforms allow modelling of barriers and interfaces of tissues as well as a tight control of forces, perfusion and strains. Interfaces can be based on synthetic materials, hydrogels or self-assembled (160, 161). Organs-on-a-chip employ a combination of all the above techniques to generate organotypic models with geometrically defined multicellular composition, mechanical/electrical/biochemical stimulation and controlled liquid flow (160, 161). Organs-on-a-chip have been generated for lungs, heart, kidney, liver, muscle, while chips recapitulating immune responses have been underexplored so far, with the notable exceptions reviewed in ref. (162).

Laser-Capture Microdissection

The introduction of laser-capture microdissection technology has been largely used to study TME over the last 15 years (163, 164). The possibility to laser-cut a piece of tumor from a tissue section and specifically isolate cells from the TME by fluorescence activated cell sorting (FACS) enormously contributed to our knowledge in the field. However, despite being a powerful methodology this has some major limitations, mostly due the quality of the isolated material from a fixed tissue. Moreover, this approach could be very complicated to adopt when the spatial location of small metastatic nodules is required. Techniques involving the labeling of stromal cells within the niche could overcome these limitations. Once labeled, these cells can be isolated as live cells by flow cytometry, allowing their functional characterization *ex vivo*. Nowadays, the possibility to couple *in vivo* labeling techniques with state-of-art single cell analysis could enormously extend what we know about the role of the TME in the coming years.

In Vivo Labeling of Metastatic Niche

In this paragraph, we will discuss in detail some recently developed *in vivo* labeling methods. These systems have potential to be optimized in the context of dormancy and may finally reveal the "dormant niche" *in vivo*.

The techniques most commonly used to identify and isolate cells from tissues, including the most recent ones we describe in this section, imply using Fluorescence-Activated Cell Sorting (FACS) during the procedure. FACS has indeed proved to be a key asset to study the TME and the use of specific cellular markers has been critical to characterise different cell populations within the TME. In **Supplementary Table 1**, we provide a list of markers that might be useful to identify the cellular populations in the TME (this list has to be considered as a simplified guide to roughly discriminate the most abundant cellular components and need to be refined according to specific experimental needs and continuously revised as new findings emerge). However, advances in new technologies, and particularly the advent of the single cell RNA sequencing (scRNA-seq), keep revealing how the expression of markers initially thought to be exclusive of one lineage population are actually shared among different cell populations. Moreover, high heterogeneity and plasticity have been observed within the same cellular components in the TME, for example in tumor-associated macrophages and cancer-associated fibroblasts among the others (165–170). All this complexity makes it difficult to distinctively isolate some cellular sub-populations. The possibility to couple unbiased niche labeling methods with scRNA-seq could help to define more precise combinations of markers to identify specific subpopulations.

The generation of a genetic mouse model expressing a photoactivatable GFP (171) coupled with the two-photon microscopy technology allowed photoactivation of specific regions of inguinal lymph nodes with a technique called NICHE-seq (**Figure 2**) (172). Labeled cells were isolated by FACS and analysed by single cell RNA-sequencing. The same approach has shown potential to photoactivate regions surrounding melanoma cells (172). The main limitation is represented by the physical accessibility of the tissue to imaging and photoactivation, and by the requirement to precisely locate the cancer cell within an entire tissue, that can be particularly challenging in the case of isolated DDCCs.

To overcome this limitation, Headley and colleagues engineered melanoma cells to express cytoplasmic Zs-green, a highly brilliant fluorescent protein, that is also incorporated in tumor cell fragments (cytoplasts). By endocyting these fragments, neighboring cells become fluorescent themselves and can be visualised or isolated (159). The efficacy of this method depends on the amount of microvesicles the tumor cells release and on the ability of the neighbouring cells to internalise/phagocyte them, therefore limiting cell detection mostly to myeloid immune cells.

In an alternative method called LIPSTIC, a receptor-ligand interaction can be marked by the transferring of a biotin-tag on the recipient cells (173). Here, "donor" T cells expressing a CD40L fused to Sortase A interacted with B-cells engineered to express an

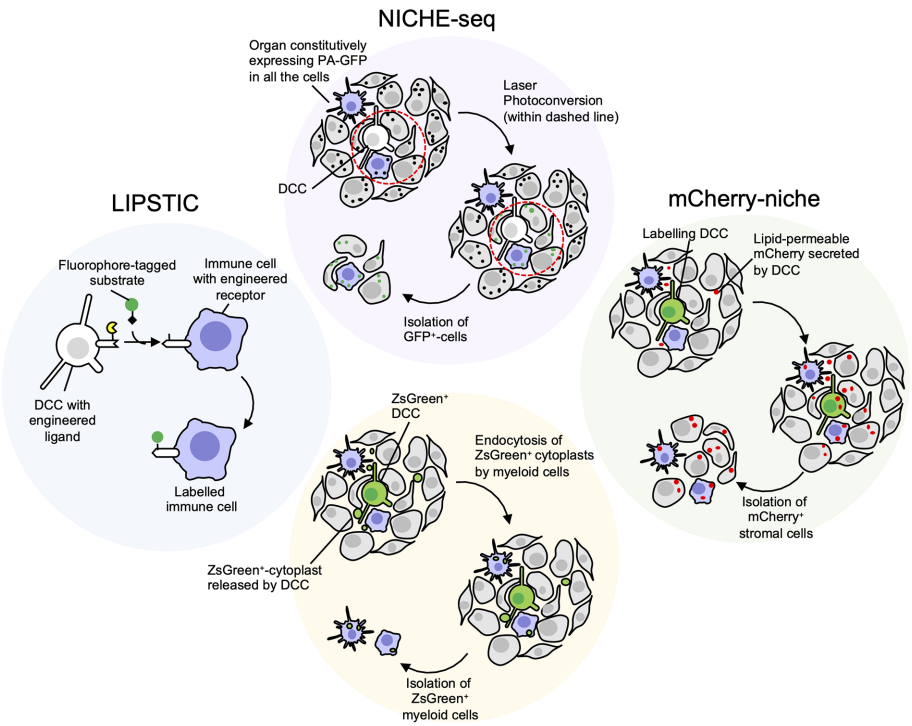

FIGURE 2 | Niche-labeling techniques for characterization of immune metastatic tumor microenvironment (TME). White/Green: cancer cells; Grey: stromal cells; Purple: immune stromal cells. NICHE-seq (172) employs transgenic mice constitutively expressing photoactivatable GFP (PA-GFP), a fluorescent protein that increases its emission after excitation with 413 nm light (black dots: dark state; green dots: fluorescent state after photoconversion). Once a disseminated cancer cell (DCC) is located in the metastatic organ the surrounding niche can be irradiated and GFP^+ cells isolated. Main limitations: i) the difficulties to spatially locate few scattered dormant DCCs (DDCCs) throughout entire organs; ii) the accessibility of those organs for photoconversion *in vivo*. The latter issue can be overcome with *ex vivo* photoconversion of freshly explanted organs. LIPSTIC (labeling Immune Partnerships by SorTagging Intercellular Contacts) (173) is an intercellular enzymatic labeling technique that exploits *Staphylococcus aureus* transpeptidase sortase A (SrtA, in yellow). Here, SrtA transfers a substrate containing "LPXTG" motif (black diamond), fused with biotin or fluorophore, to five N-terminal glycine residues tag (G5). This transfer requires proximity of SrtA and G5, thus a receptor and its membrane-bound ligand are fused with either SrtA and G5 in different cells. If these cells, that could be DDCCs and stromal cells, lie in close proximity at the metastatic site, stromal cells surrounding DDCCs are labeled and can be isolated for further analysis. Main limitations: i) the stromal lineage of interest must be genetically engineered *a priori* with tagged receptor or ligand, making this technique not suitable for unbiased identification of niche stromal cells; ii) the cells must be in close proximity for the reaction to happen. Cherry-niche (172) was developed to overcome these limitations. Here, the mCherry protein is engineered with a lipid-permeable domain (sLP-mCherry). DCCs expressing the sLP-mCherry release the protein in the extracellular space and the protein is uptaken by neighboring cells that can be isolated and analysed. Similarly, Krummel lab observed that blebs (cytoplasts, green dots) released by disseminated $ZsGreen^+$-melanoma cells are endocytosed by resident myeloid cells (159). This approach is limited to DCCs releasing a significant amount of blebs and to stromal cells with efficient endocytic capacity.

"acceptor" domain fused to the CD40 receptor. When the receptor-ligand interaction occurs in presence of a fluorescent or a biotinylated substrate, the acceptor cells are labeled. This strategy implies a physical interaction between cells, and a ligand-receptor pair previously engineered and expressed by the right cell lineage(s). Moreover, the ectopic expression of endogenous ligand-receptors may cause unwanted biological effects, thus suggesting the need to engineer more neutral synthetic systems.

Another approach that we have recently developed, named Cherry-niche, allows engineered cancer cells to label their surroundings by transferring a modified red-fluorescent protein (174). Neighbouring cells of the cancer cells endocyte this protein and become fluorescent. Thanks to its liposoluble features, Cherry-niche does not require direct cell-cell contact nor a-priori knowledge of the recipient cells, as all the surrounding cells have the potential to internalise the fluorescent tag. Importantly, in *in vivo* organs, such as in the lung, the bulk of the labeling is limited to the close proximity of the metastatic cells, highlighting the potential of Cherry-niche to specifically reveal the cancer neighbouring cells.

CONCLUSIONS

In the last decade tremendous advancements have been achieved in oncology following the development of cutting-edge techniques. Among the different aspects of cancer biology, survival, quiescence and outgrowth of DCCs remained underexplored due to experimental hurdles such as faithfully modeling of metastatic organs *in vitro* and labeling of metastatic TME *in vivo*. In this

review we presented recent techniques that in our opinion will give great impulse towards these directions. Despite this, our knowledge of DDCCs in human patients is extremely limited. This is mostly due to the current lack of techniques to track single or small clusters of DDCCs, together with ethical and technical issues with collecting and analysing metastatic organs in cured healthy patients. A notable exception is the bone marrow, a frequent site of relapse for several cancers. From this tissue, single DDCCs have been isolated, profiled (175) and provided clinical evidence of the existence of DDCCs in patients with no evidence of disease (176, 177). Isolation of circulating tumor cells or circulating tumor-derived factors from blood biopsies holds great potential to bypass the aforementioned limitations (178), although work is still needed to identify DCCs with metastasis-forming ability from a heterogeneous population of DCCs. Moreover, DDCCs do not effectively respond to chemotherapies or radiation therapies as a consequence of quiescence and because of the protective role of microenvironment (45, 179), thus, immunotherapy holds great hopes for clearing organs from DDCCs before relapse. In the light of this, it will be of utmost importance to exploit the most recent techniques to deepen our knowledge of DDCCs-immune cells crosstalk at the metastatic site.

AUTHOR CONTRIBUTIONS

LO and MM equally contributed to conceiving and writing the review. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.594514/full#supplementary-material

SUPPLEMENTARY TABLE 1 | Lineage markers to identify cell populations in the murine TME. TME components have been roughly categorised here in broad stromal cell types (immune, endothelial, mesenchymal and parenchymal). CD45 can be used to identify all the immune cells. To further discriminate subpopulation within the immune compartment it needs to be combined with other markers. For example, a combination of CD45, CD11b and Ly6G will distinctively identify the neutrophils. This list represents a very simplified guide to roughly discriminate the most abundant cellular components in the TME, therefore not including all type of cells that might be found. We recommend to refine the marker combination to use according to specific experimental needs. Moreover, new findings may need to be considered to implement or update this list.

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REVIEW
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Inflammation-Driven Breast Tumor Cell Plasticity: Stemness/EMT, Therapy Resistance and Dormancy

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Cellular heterogeneity poses an immense therapeutic challenge in cancer due to a constant change in tumor cell characteristics, endowing cancer cells with the ability to dynamically shift between states. Intra-tumor heterogeneity is largely driven by cancer cell plasticity, demonstrated by the ability of malignant cells to acquire stemness and epithelial-to-mesenchymal transition (EMT) properties, to develop therapy resistance and to escape dormancy. These different aspects of cancer cell remodeling are driven by intrinsic as well as by extrinsic signals, the latter being dominated by factors of the tumor microenvironment. As part of the tumor milieu, chronic inflammation is generally regarded as a most influential player that supports tumor development and progression. In this review article, we put together recent findings on the roles of inflammatory elements in driving forward key processes of tumor cell plasticity. Using breast cancer as a representative research system, we demonstrate the critical roles played by inflammation-associated myeloid cells (mainly macrophages), pro-inflammatory cytokines [such as tumor necrosis factor α (TNFα) and interleukin 6 (IL-6)] and inflammatory chemokines [primarily CXCL8 (interleukin 8, IL-8) and CXCL1 (GROα)] in promoting tumor cell remodeling. These inflammatory components form a common thread that is involved in regulation of the three plasticity levels: stemness/EMT, therapy resistance, and dormancy. In view of the fact that inflammatory elements are a common denominator shared by different aspects of tumor cell plasticity, it is possible that their targeting may have a critical clinical benefit for cancer patients.

Keywords: cytokines/chemokines, dormancy, epithelial-to-mesenchymal transition, inflammation, macrophages, stemness, therapy resistance, tumor cell plasticity

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INTRODUCTION

The global effort to develop improved therapies for cancer patients is an enduring task, partly due to tumor cell heterogeneity that characterizes many different cancer types. The reasons for heterogeneity are diverse and include among others the constant transition of tumor cells between different phenotypic and functional states. Assembled together under the term "cancer cell plasticity", various types of transition events take place at primary tumors and in metastatic foci (1–8).

Cancer cell heterogeneity and plasticity are well-documented in breast cancer (BC), where they contribute to immense mechanistic and functional complexity, and have cardinal therapeutic implications (4, 9, 10). As they manifest inter-tumor heterogeneity, breast tumors are categorized in four groups that are differently treated in the clinic, based on the expression of estrogen receptors (ERs), progesterone receptors (PRs) and HER2: luminal-A cancers, where tumors express ER/PR and are HER2-negative (HER2−); luminal-B tumors that are positive for ER/PR and demonstrate HER2 amplification (HER2+) or are ki67-high; HER2+ tumors that do not express ER/PR and demonstrate HER2 over-expression; and triple-negative BCs (TNBCs) that lack the expression of the three receptors: ER, PR and HER2 (11–13).

In parallel to inter-tumor heterogeneity, intra-tumor heterogeneity is also highly apparent in BC. Breast tumors can include cancer stem cells (CSCs) and non-CSCs as well (4, 9, 10); some of the cancer cells undergo epithelial-to-mesenchymal transition (EMT) while others do not (10); under certain conditions, cells that develop acquired resistance to different types of therapy are present among the cancer cells (4, 14); and in addition, cancer cells can gain the ability to remain dormant as single or clustered micro-metastasizing cells or exhibit through different modifications the ability to exit dormancy (4, 15).

These different heterogeneity facets are often linked to each other, as seen for example by the connection between CSC and EMT states, by the fact that CSCs are highly resistant to chemotherapy and by the regulation of CSC/EMT and dormancy by chemotherapy (1–4). Also, they exemplify the high degree of plasticity that characterizes cancer cells. Non-CSCs can turn to CSCs and *vice versa* (1, 5, 6); cells that have undergone EMT can more efficiently metastasize and then colonize better the metastatic niche if they have completed the opposite process of mesenchymal-to-epithelial transition (MET) (5); therapy-resistance is subject to alterations that have a strong impact on the well-being of patients and their survival, and tumor cells can adapt to stress by entering a dormant phase but can also escape dormancy when conditions change (2, 3, 6).

This dynamic remodeling of the cancer cells depends on cell-autonomous traits (*e.g.* epigenetics, metabolism, endoplasmic reticulum stress) but in parallel the tumor microenvironment (TME) has substantial ability to shape the phenotypes and functions of the cancer cells and thus dictates the degree of cancer heterogeneity and plasticity (16). In this context, major roles were recently attributed to immune/inflammatory cells and to the factors that mediate their activities (17, 18). The diversity of immune cells and the balance between the acquired immunity arm and the inflammatory arm have prominent impacts on the fate of the tumor and its progression. When acquired immunity is concerned, it is well known that specific cell types, like T helper 1 (Th1) cells and cytotoxic T cells (CTLs) are key players in immune surveillance and their activities may lead to cancer cell eradication; these effects may be strengthened by immunotherapies (*e.g.*, those directed to inhibitory immune checkpoint molecules like PD-1, PD-L1, and CTLA-4) and contribute to tumor suppression (19, 20). However, acquired immunity may also have opposing effects, as demonstrated for example by the ability of Th1-derived interferon γ (IFNγ) to up-regulate inhibitory checkpoint molecules (21), while Th2 cells can release cytokines that divert macrophages to an M2 phenotype that supports tumor progression (22). As expected, different aspects of cancer cell plasticity were shown to be regulated by cells and factors of acquired immunity [*e.g.*, (17, 18, 23, 24)]; however, in view of the many facets of acquired immunity in malignancy, this topic will not be addressed in depth in this review.

In parallel to acquired immunity, myeloid cells and pro-inflammatory products exert a large variety of effects that contribute to increased metastasis and reduced survival, mainly at more advanced stages of tumor progression. Regarded as "The seventh hallmark of cancer", cancer-related inflammation has a very strong impact on disease progression, contributing to tumor development and metastasis (25–28). In view of their critical functions at the TME and their significant impacts on disease course, it is no surprise that inflammatory elements also regulate many cardinal aspects of tumor cell remodeling, as discussed and illustrated in this review article (summarized in **Figure 1** and **Tables 1**–**3**).

Herein, we focus on the inflammatory arena and its impacts on cancer cell plasticity. To demonstrate the activities of inflammatory players in controlling tumor cell remodeling, we highlight their roles in dictating the dynamic nature of tumor cells at the following three major levels of plasticity: (1) Stemness and EMT; (2) Resistance to different types of therapy; (3) Entry to and exit from dormancy (1–10).

In addressing these three plasticity-related topics, we hereby focus on breast malignancy in order to exemplify the roles of three key inflammatory axes:

(1) Inflammation-associated myeloid cells: Breast tumors are usually characterized by pronounced chronic inflammation and are enriched with different types of myeloid cells. Here, major roles are attributed to tumor-associated macrophages (TAMs) in promoting cancer progression (22, 29). TAMs are typically regarded as alternatively-activated M2 macrophages, but under certain conditions macrophages with a classically-activated M1 phenotype can also have significant roles in promoting cancer progression (30–32).

(2) Pro-inflammatory cytokines: Here, we put major emphasis on tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) and the canonical transcription factors that mediate their activities: NF-κB and STAT3, respectively (25, 33–35). The chronic presence of TNFα in tumors strongly enhances tumor progression (36–40); in parallel, IL-6 is also considered a strong tumor-promoting factor (41–43). These elements are well characterized for their ability to enhance tumor progression in many malignancies including BC (35, 36, 38, 41–43).

(3) Inflammatory chemokines: Consisting of four structural sub-groups—of which the CXC and the CC are the largest—and homeostatic and/or inflammatory activities, chemokines are key players in protection against pathogens but also have cardinal roles in regulating malignancy (44–54). Inflammatory chemokines of the ELR+ CXC sub-group contribute immensely to tumor-related inflammation; they chemoattract

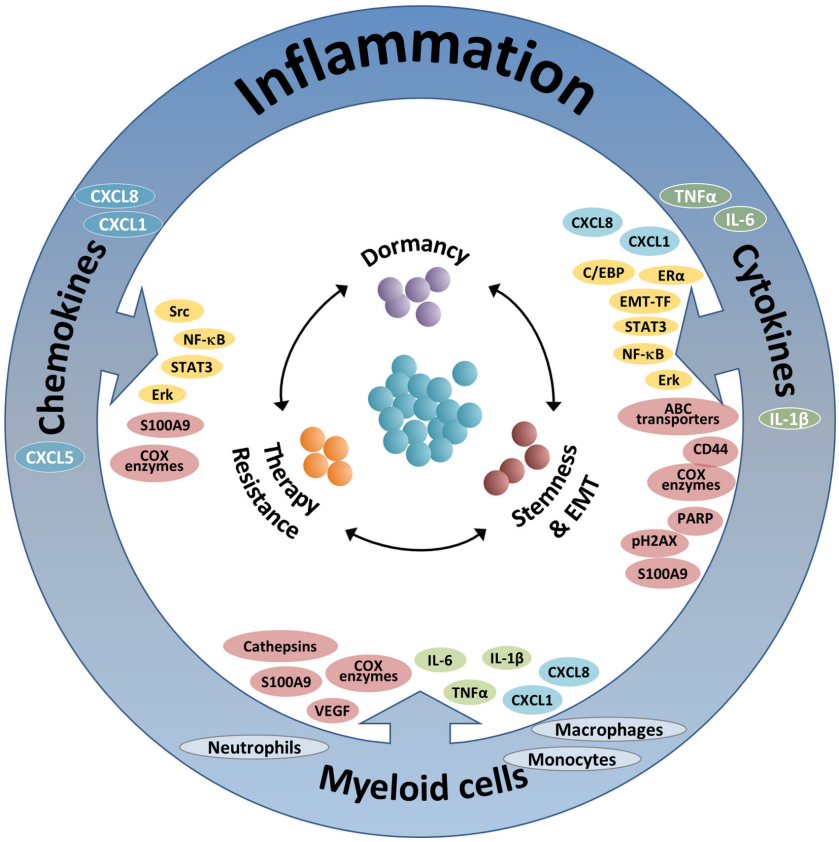

FIGURE 1 | The integrated activities of pro-inflammatory cells and soluble pro-inflammatory factors in controlling tumor cell plasticity. The Figure demonstrates the roles of three inflammatory axes in regulating key aspects of cancer cell plasticity: stemness/EMT, therapy resistance and dormancy (see also **Tables 1**–**3**). Based on the findings summarized in this review, it is proposed that therapeutic modalities that target such key inflammatory elements will affect simultaneously several modes of malignant cell plasticity and will therefore provide improved approaches of cancer therapy. The three inflammatory axes addressed in this manuscript include: (1) Inflammation-associated myeloid cells: Here, the main contributors to regulation of tumor cell plasticity are macrophages and monocytes, and also neutrophils. These cells control tumor cell remodeling by forming physical contacts and also by exchanging soluble mediators with the cancer cells. The major elements involved in the activities of myeloid cells include: In green—Pro-inflammatory cytokines; In cyan—Inflammatory chemokines; In brown—Other intracellular components. (2) Pro-inflammatory cytokines: In this context, major roles are attributed mainly to the pro-inflammatory cytokines TNFα and IL-6 (and to some extent also IL-1β), whose activities are mediated mainly by activation of transcription factors (TFs) and up-regulation of inflammatory chemokines. The main elements involved in this axis include: In yellow—Inflammation-related TFs and other TFs; In cyan—Inflammatory chemokines; In brown - Other intracellular components. (3) Inflammatory chemokines: Here, mainly chemokines of the ELR+ CXC sub-group are implicated, primarily CXCL8, CXCL1 and CXCL5; other chemokines such as CCL2 and CCL5 are also involved, as discussed in brief in the manuscript (not shown). In this setting, the major elements involved include: In yellow—Inflammation-related TFs and other TFs; In brown—Other intracellular components.

leukocytes with pro-malignancy roles to tumors and they can also induce angiogenesis and act directly on the cancer cells, to promote their metastatic potential (44–54). Thus, in this review we focus on inflammatory ELR+ CXC chemokines, such as CXCL8 (interleukin 8, IL-8) and CXCL1 (GROα), and we also address their respective receptors: CXCR2 (for both chemokines) and CXCR1 (for CXCL8) (52, 55–57). Occasionally, we also describe the involvement of other members of the family of inflammatory chemokines, such as CCL2 (MCP-1) and CCL5 (RANTES).

The different players that will be addressed in this manuscript —myeloid cells and inflammatory factors—are strongly linked to each other at many different manners, when pathogen-related conditions are concerned. For example, macrophages are a major source for TNFα, IL-6 and some of the chemokines (58, 59), and TNFα is a very strong inducer of many inflammatory chemokines (40, 60–63). Similarly, also in the tumor plasticity field, these different inflammatory components often play cooperative and simultaneous roles in promoting tumor cell stemness/EMT, therapy resistance, and dormancy in BC, as will be illustrated below.

INFLAMMATION-DRIVEN STEMNESS AND EMT IN BREAST MALIGNANCY

To successfully complete the consecutive process of tumor cell dissemination, followed by seeding and growth in metastatic niches, cancer cells need to exhibit improved capabilities to cope

TABLE 1 | Inflammation-driven cellular and molecular mechanisms regulating stemness and EMT.

STEMNESS and EMT	
Inflammatory element involved	**Main observations**
Myeloid cells	
Monocytic cells/ Macrophages/TAMs	* *Via* physical contacts with cancer cells and through secreted factors, they increased CSC proportions, as well as EMT and migration. * The activities of the myeloid cells were mediated by or connected to soluble factors such as TNFα, IL-6 and ELR+ CXC chemokines (*e.g.*, CXCL8 and CXCL1).
Soluble and cellular mediators	
TNFα, IL-6, IL-1β, ELR+ CXC chemokines	* Inflammatory cytokines sustained CD90+ CSCs in tumor cells, and had a profound ability to increase tumor cell stemness, EMT and migration. * The soluble mediators induced predominantly the activation of NF-kB and STAT3. Activation of HER2/EGFR-Src was also described. * Regulation of EMT-related transcription factors was induced by the soluble mediators, or was linked to their activity.

The table summarizes the cellular and molecular components that are involved in inflammation-driven regulation of stemness and EMT. To enable a unifying view of the findings described in different research systems, the table focuses on the major observations described in the text, related to the functions of myeloid cells, pro-inflammatory cytokines and ELR+ CXC chemokines (without CC chemokines).

TABLE 2 | Inflammation-driven cellular and molecular mechanisms regulating therapy resistance.

THERAPY RESISTANCE	
Inflammatory element involved	**Main observations**
Chemoresistance	
Myeloid cells	
Macrophages, CD11b+Gr1+ cells	* Myeloid cells released soluble factors that promoted chemoresistance, with evidence to increased proportions of chemoresistant ALDH+ CSCs. * The mediators involved in chemoresistance included mainly TNFα, cathepsins and S100A9.
Soluble and cellular mediators	
TNFα, mTNFα, ELR+ CXC chemokines, cathepsins, S100A9	* TNFα was directly active in promoting chemoresistance, in its soluble and membrane forms. * TNFα has induced the expression of chemokines that recruited myeloid cells to tumors. * ELR+ chemokines directly promoted chemoresistance, for example by elevating tumor cell viability in a S100A9-dependent manner. * Cathepsins protected the cancer cells from chemotherapy. * The molecular elements involved in chemoresistance included primarily NF-kB and STAT3 activation, and also activation of C/EBP and of the Lin-28B-Let7-HMGA2 axis. * Inhibition of pH2AX expression and elevated expression of PARP were involved in chemoresistance, as well as elevated expression of ABC transporters.
Endocrine resistance	
Myeloid cells	
Macrophages	* Through the release of soluble mediators, macrophages have led to resistance to estrogen withdrawal and to tamoxifen resistance. * The soluble mediators involved included mainly TNFα, IL-6 and chemokines.
Soluble and cellular mediators	
TNFα, IL-6, ELR+ CXC chemokines	* The soluble mediators reduced the expression of ERα or have led to its constitutive activation. * The molecular mechanisms included activation of the NF-*k*B and STAT3 pathways, down-regulation of FOXO3a and involvement of COX-2.

The table summarizes the cellular and molecular components that are involved in inflammation-driven regulation of therapy resistance (addressing here only chemotherapy and endocrine therapy) and dormancy. To enable a unifying view of the findings described in different research systems, the Table focuses on the major observations described in the text, related to the functions of myeloid cells, pro-inflammatory cytokines and ELR+ CXC chemokines (without CC chemokines).

with the obstacles they encounter along the way. When tumor heterogeneity is closely examined, it is possible to identify cells that have acquired stem cell characteristics and/or underwent EMT, thus demonstrating plasticity that contributes to their ability to better handle the challenges they face during metastasis (64–69).

BC cells clearly follow the same paradigm, demonstrating dynamic remodeling that leads to development/selection of cancer stem cells (CSCs) and of tumor cells that have undergone EMT. More so, similar to other cancer cell types, also in BC the two processes are closely linked, as manifested for example by the fact that CSCs often express EMT characteristics

TABLE 3 | Inflammation-driven cellular and molecular mechanisms regulating tumor cell dormancy.

DORMANCY	
Inflammatory element involved	**Main observations**
Myeloid cells	
Macrophages, monocytes, neutrophils	* Myeloid cells were connected to tumor recurrence following surgery and to exit from dormancy. * Myeloid cells acted in this respect *via* soluble mediators like TNFα, IL-1β and VEGF-A, and through COX enzymes.
Soluble and cellular mediators	
TNFα, IL-1β, IL-6, ELR+ CXC chemokines, COX enzymes	* Inflammatory cytokines increased the proliferation of dormant cells, including at the bone niche. * TNFα induced chemokine expression *via* the IKK-NF-*k*B pathway, and together with other pro-inflammatory cytokines has led to increased tumor cell proliferation and exit from dormancy. * STAT3 was also found to be a key regulator of cytokine activities in dormancy (its activities depended on the ligand used). * Stimulation of CXCR2 by its cognate ELR+ chemokines has led to Erk signaling; it increased cancer cell proliferation under quiescent conditions and promoted emergence from chemotherapy-induced dormancy. * COX-2 has led to increased aromatase expression, leading to elevated ERα activity and tumor cell proliferation.

The table summarizes the cellular and molecular components that are involved in inflammation-driven regulation of cancer cell dormancy. To enable a unifying view of the findings described in different research systems, the Table focuses on the major observations described in the text, related to the functions of myeloid cells, pro-inflammatory cytokines and ELR+ CXC chemokines (without CC chemokines).

(67–69). Under these conditions, BC cells benefit from the advantages provided by these two properties together and express higher ability to metastasize and resist different therapeutic modalities (67–69).

In the context of cancer cell plasticity, stemness and EMT processes have a major influence on the functional state of cancer cells. Stemness in BC is mainly identified by increased presence of CD44+/CD24− cells or CD44+/CD24− low cells, and ALDH+ cells; by the expression of stemness genes and/or by the ability of the tumor cells to form mammospheres (tumor spheres). CSCs are also greatly connected to increased therapy resistance, particularly in response to chemotherapy (5, 9, 70, 71).

In parallel, EMT is characterized by the acquisition of mesenchymal morphology; reduced expression of E-cadherin; increased expression of N-cadherin, vimentin and/or fibronectin; and elevated expression of EMT regulators such as twist, snail, slug and zeb (5, 10, 70, 71). As expected, the EMT process is often accompanied by elevated migratory and invasive properties of the cancer cells (70, 71).

As illustrated below, inflammatory cells and soluble mediators have prominent roles in regulating stemness and EMT in breast cancer, often with interactions between the different players (summarized in **Figure 1** and **Table 1**).

Inflammation-Associated Myeloid Cells Regulating Stemness and EMT

Macrophages form an important hub, converging the functions of many regulators of stemness and EMT. The impact of macrophages and of the factors they release on these remodeling processes were revealed by studies of monocytic cell co-cultures with breast tumor cells and by investigations in which cancer cells were grown in the presence of macrophage-derived conditioned media (CM). Under both conditions, of direct or indirect interactions between the cancer cells and monocytic cells, elevated levels of stemness, EMT or both were frequently mediated by inflammatory factors such as TNFα, IL-6 and/or ELR+ CXC chemokines that will be particularly mentioned in more detail later on.

For example, a research by Weinberg and his colleagues demonstrated that in BC patient biopsies, CD68+ macrophages were localized in proximity to CD90+ tumor cells, which were endowed with characteristics of CSCs as well as EMT (72). In this report, macrophages enhanced tumor initiation by CD90+ CSC cells and promoted the formation of primary tumors and of metastases (72). Of interest was the fact that tumor cell-expressed CD90 was required for generating physical contacts between the tumor cells and macrophages, and these interactions have increased the expression of IL-6, CXCL8 and granulocyte-macrophage colony stimulation factor (GM-CSF) in the cancer cells. In parallel, inflammatory cytokines sustained the levels of CSCs, and recombinant IL-6 and CXCL8 increased the formation of tumor spheres by CD90+ tumor cells (72).

When considering the type of macrophages that regulate stemness/EMT, it is interesting to note that when CM of M1 cells (mainly those derived from peripheral blood mononuclear cells, PBMCs) have been added to luminal-A BC cells, the tumor cells have acquired a CSC phenotype (elevated formation of mammospheres), EMT properties (mesenchymal morphology, reduced E-cadherin and elevated zeb1 expression) and more potent migration (73). By adding neutralizing antibodies to specific pro-inflammatory cytokines, the researchers demonstrated that TNFα, interleukin 1β (IL-1β) and IL-6 partly contributed to some of these effects. In line with the fact that M1-derived CM have led to STAT3 activation, inhibition of the Jak2-STAT3 pathway alongside with NF-κB down-regulation resulted in reduced EMT properties and lower proportions of CD44+/CD24−/low CSCs (73).

In another investigation it was found that inflammatory BC (IBC) tumors were significantly infiltrated by CD163+ M2 macrophages; moreover, IBC cells in culture released chemokines that induced the recruitment of macrophages, as well as factors that induced macrophage polarization to the M2 direction (74). In this study, which has used THP-1 cells or blood monocytes, physical co-culturing of monocytic cells with cancer cells has given rise to elevated proportions of CSCs (CD44+/CD24− and ALDH+) and the cancer cells have gained some EMT characteristics (74). Some of these EMT phenotypes and tumor cell invasion were up-regulated by ELR+ CXC chemokines that were present in the culture medium of the macrophages, such as CXCL8 and CXCL1 (74). In this case, the chemokines have led to STAT3 activation in the tumor cells, which then has driven forward stemness and EMT (74).

In the same spirit, in response to CM of blood monocyte-derived macrophages, several breast tumor cell lines acquired a stronger invasive capability; in MCF-7 cells, this effect was mediated by TNFα-induced stabilization of snail, mediated by the ability of the cytokine to activate NF-κB (75). Moreover, in a study using TAMs excised from a MMTV-PyMT tumor model and monocytic cells, the overall conclusion was that macrophages released CXCL1, which *via* NF-κB activation has induced the transcription of the EMT regulator SOX4, leading to EMT and metastasis (76). It is interesting to note that in this study, CXCL1 was not found to induce the enrichment of CSCs (76).

Pro-Inflammatory Cytokines Regulating Stemness and EMT

The above studies demonstrated that contacts formed between monocytic cells and BC cells, as well as factors released by macrophages had a major role in promoting stemness and EMT in BC. As mentioned, the factors involved in macrophage-mediated regulation of stemness and EMT included pro-inflammatory factors such as TNFα, IL-1β, IL-6 and the ELR+ CXC chemokines CXCL8 and CXCL1. In parallel, in other studies, these soluble factors were found to promote the proportions of CSCs and/or of cells undergoing EMT independently of macrophage-related aspects.

For example, the roles of TNFα were studied extensively in BC by using it in a recombinant form or by employing CM of TNFα-stimulated cells. In many studies, TNFα has directly induced stemness, EMT, and migration in BC cells and in non-transformed breast epithelial cells; in this regard, TNFα acted when it was used alone or when it was joined by other TME factors that often potentiated its activities, such as the EMT-inducer transforming growth factor β (TGFβ) or estrogen + epidermal growth factor (EGF) (40, 62, 63, 77–88). In some of the settings, it was the extended stimulation of cells by TNFα for up to several weeks that has led to pronounced effects on stemness as well as on EMT (77–79). Mechanistically, TNFα activities were accompanied by modified expression of CSC markers (like elevated presence of CD44+/CD24− or CD44+/CD24− low cells) and elevated expression of zeb1, slug and/or twist1. Often, these TNFα-induced functions were connected to activation of the NF-κB pathway (77, 79, 80, 82, 83, 88).

Adding to reports describing the roles of TNFα in stemness/EMT induction in BC, a large number of studies indicate that IL-6 is a major inducer of stemness in this disease (72, 73, 89–96). IL-6 was found to promote stemness mostly in transformed but also in non-transformed breast epithelial cells. Many of these investigations have demonstrated the roles of IL-6 in promoting stemness by using a recombinant cytokine (73, 89–92); others have shown autocrine sources of IL-6, or demonstrated that the cytokine was released during tumor cell interactions with other cells (such as macrophages) (72, 73, 93, 95, 96). In line with the key roles of IL-6 in enhancing tumor cell stemness, the JAK-STAT3 pathway was connected to or proven to take part in these events (73, 90, 93–95); also, the Notch pathway was suggested to mediate IL-6-induced stemness in BC (91, 97).

Inflammatory Chemokines Regulating Stemness and EMT

In addition to the pro-inflammatory cytokines TNFα and IL-6, chemokines such as CCL2, CCL5, and CXCL12 can regulate stemness and EMT in BC (reviewed in (50)). In parallel, strong impacts were found for ELR+ CXC chemokines in regulation of these aspects of plasticity in BC (50). Here, CXCL8 was demonstrated to act in autocrine and paracrine manners, depending on the research system used. The contribution of CXCL8 to stemness/EMT was demonstrated by determining the effects of recombinant CXCL8 (72, 74, 89, 98–100) and/or by using siRNA/neutralizing antibodies directed to the chemokine (74, 89, 96, 101–103). In such studies, CXCL8 was noted in CM of breast tumor/senescent cells or was induced by chemotherapy and by different stimuli such as over-expression of the transcription factor Brachyury (89, 96, 100, 101, 104). In parallel, several reports focused on the roles of the CXCL8 receptors CXCR1 and CXCR2 in controlling stemness and EMT in BC (99, 100, 104, 105). For example, inhibitors of CXCR1/2 reversed CXCL8-induced mammosphere formation by normal breast epithelial cells and by patient-derived BC cells (99, 105). In the latter study, the effect of the CXCL8–CXCR1/2 axis on stemness was shown to be through the transactivation of HER2 and EGFR by Src (105). Inhibitors of CXCR1/2 also showed that these receptor/s controlled the expression of EMT markers and induced elevations in tumor cell invasion (100, 103).

In parallel to CXCL8, CXCL1 also contributed to stemness (74, 106), but its impacts were mostly found in regulation of EMT-related processes, migration and invasion; in this respect, CXCL1 was found to act in an autocrine manner and/or to be secreted by macrophages in vicinity of the cancer cells (74, 76, 106, 107). Mechanistically, the involvement of several different signaling pathways in CXCL1-induced EMT processes was noted: NF-κB (76), STAT3 (74) and also the MAPK pathway (107), proposing there is more than one molecular pathway linking CXCL1 stimuli with different EMT characteristics.

INFLAMMATION-DRIVEN THERAPY RESISTANCE IN BREAST MALIGNANCY

Therapeutic options given to BC patients depend on tumor subtype and clinical parameters, and their efficacy is often

reduced because of intrinsic or acquired resistance. The molecular mechanisms enabling cancer cells to withstand chemotherapy, endocrine therapy, targeted therapy and immunotherapy are diverse and complex (2–4, 6). They represent, between others, selection of cells that underwent dynamic remodeling and gained the ability to subvert the effects of treatment (2–4, 6).

In the context of therapy resistance, inflammatory mechanisms are most influential and play key roles in shaping the nature and extent of therapy-relevant alterations taking place in the cancer cells (summarized in **Figure 1** and **Table 2**).

Chemoresistance: Inflammation-Associated Myeloid Cells and Pro-Inflammatory Cytokines/Chemokines

Elevated levels of macrophages were detected in post-chemotherapy biopsies of BC patients compared to pre-chemotherapy samples; increased macrophage presence was also found in BC animal model systems following taxol treatment (108). In this research it was also demonstrated by *in vitro* studies that macrophage-derived cathepsins have protected the tumor cells from taxol-induced cell death, and that cathepsins also reduced the efficacy of taxol *in vivo* (108). In another research, macrophages were connected to chemoresistance when antibodies targeting colony-stimulating factor-1 (CSF-1), a major monocyte chemoattractant, increased the efficacy of chemotherapy in reducing tumor sizes *in vivo*, in a process that was accompanied by lower macrophage presence in tumors (109). Phenotypically, it was found that M1 macrophages secreted factors that have led to elevated proportions of ALDH+ CSCs through NF-κB and STAT3, and *via* the Lin-28B-let-7-HMGA2 axis; these CSCs expressed increased resistance to doxorubicin, 5-FU and paclitaxel (73).

In parallel, another study demonstrated key roles for the CD11b+Gr1+ myeloid sub-population in mediating resistance to chemotherapy in BC. These cells were recruited to tumors by cancer cell-derived CXCL1/2 chemokines, and served as a major source for the pro-inflammatory proteins S100A8 and S100A9. In turn, S100A9 was found to be responsible for increased survival of the cancer cells in mice treated by doxorubicin and cyclophosphamide (110). Accordingly, analysis of BC patient biopsies demonstrated increased expression of S100A8/9 following chemotherapy (110). The findings of this study also indicated that the CXCL1/2–S100A8/9 axis was reinforced by chemotherapy through a stroma-derived TNFα-mediated process (110). TNFα roles in inducing therapy-resistant breast CSCs were further demonstrated when it was found that extended exposure of BC cells to combined TNFα+TGFβ stimulation has led to generation of CSCs that acquired high levels of resistance to chemotherapy, accompanied by elevated expression levels of ABC transporters (84).

Being a part of the complex network of TNFα and its ligands, the transmembrane form of TNFα (tmTNFα) was found to be expressed at high levels in a considerable proportion of BC patient tumors, mainly of the TNBC subtype (111). By using primary tumor cells, it was demonstrated in this study that high expression levels of tmTNFα were correlated with elevated resistance to anthracycline (111). Making use of shRNA to TNFα and the N-terminal fragment of tmTNFα, the authors of this investigation have concluded that reduced expression of tmTNFα improved the sensitivity of breast tumor cells to doxorubicin, and that the activities of tmTNFα in resistance were meditated by NF-κB and Erk (111). In addition, a recent study concerning the TNFα receptor TNFR2 in TNBC and luminal-A BC cells indicated that its down-regulation has improved the efficacy of adriamycin; TNFR2-mediated resistance was taking place through inhibition of adriamycin-induced pH2AX expression, mediated by enhanced expression of poly(ADP-ribose) polymerase (PARP) (112).

Throughout these investigations, some insights were provided to molecular mechanisms that may mediate inflammation-driven processes of chemoresistance. Here, major roles were attributed to the transcription factor NF-κB (103, 111, 113, 114). Particularly, a recent study that has analyzed cross-tolerance of breast tumor cells to anthracyclines (doxorubicin), which has developed in taxane (doxetaxel)-resistant cells, indicated that inflammatory cytokines and NF-κB were involved in the process (114); this investigation has indicated that GM-CSF + IL-23 + IFNγ, as well as NF-κB-mediated signaling induced the expression of CD44 by taxane-resistant cells, initiating signaling and metabolic cascades that regulated cross-tolerance (114). In addition, STAT3 was strongly implicated in reducing the sensitivity of breast tumor cells to chemotherapies. In line with the fact that STAT3 is a key transcription factor mediating the effects of IL-6 (42, 115), direct roles were reported for IL-6 in inducing chemoresistance, which was mediated by activation of C/EBP (116).

However, irrespectively of IL-6 activities, STAT3 involvement in chemotherapy resistance was proposed by a number of publications. For example, in doxorubicin-resistant TNBC cell inhibition of STAT3 activation by pharmacological inhibitor partly restored cancer cell sensitivity to the treatment, possibly through reduction in CSC proportion (117). In another study it was found that IL-22—whose expression by T cells was increased in TNBC tumor tissues compared to para-tumor and normal areas—induced paclitaxel resistance in TNBC cell lines, accompanied by elevated JAK/STAT3 activation (118). The JAK/STAT3 pathway was also found to regulate leptin-induced fatty acid β-oxidation, promoting self-renewal and chemoresistance in breast CSCs (119). This latter study also reported that STAT3 mRNA levels were higher in *ex vivo* cultured BC tumors derived from post-chemotherapy TNBC biopsies compared with pre-chemotherapy tumor tissues (119).

As part of the mechanistic analyses included in the above studies, the chemokines CXCL1/2 were found to be involved in regulation of chemoresistance (110). The roles of such chemokines in this process are strongly supported by additional investigations addressing inflammatory ELR+ CXC chemokines and their receptors in inducing or mediating lower sensitivity levels to chemotherapies. Different studies indicated that various types of chemotherapy share the ability to promote the release of ELR+ CXC chemokines—CXCL1, CXCL2, CXCL3,

CXCL5, CXCL7 and CXCL8 (depending on the study) —by myeloid cells, breast tumor cells and mesenchymal stem cells (103, 104, 110, 120, 121). In parallel, inhibition of CXCL8 or of CXCR1/2 has increased the sensitivity of breast tumor cells to chemotherapeutic drugs, accompanied by reduced proportion of CSCs, angiogenesis, tumor growth and/or metastasis, with roles attributed to cyclooxygenase 2 (COX-2) in this process (103, 104, 110, 113, 121, 122). These findings have a high clinical relevance in view of the fact that chemotherapy has led to increased CXCR2 expression in BC patients; moreover, increased CXCR1/CXCR2 and CXCL8 expression levels were significantly correlated with poor overall and disease-free survival in studies of the TCGA dataset and of patient biopsies (103, 122).

Resistance to Other Therapies: Inflammation-Associated Myeloid Cells and Pro-Inflammatory Cytokines/ Chemokines

In a way similar to chemotherapy, recent lines of evidence indicate that inflammatory components controlled also resistance to other types of therapy in BC. For example, TNFα and/or IL-6 were strongly connected to endocrine resistance in luminal-A breast tumors, in patients as well as in model systems of cultured cells or mice (123–126). The study of tumors of ER+ HER2- BC patients revealed that resistance to tamoxifen was significantly associated with the presence of CD163+ macrophages in tumors (123). High macrophage counts were also connected to poor outcome in ER+ patients in other studies (125, 127) and macrophages were found to release factors that promoted endocrine resistance, such as TNFα; here, TNFα acted by inducing down-regulation of FOXO3a, leading to ERα down-regulation (125). In another study, TNFα was found to induce in macrophages the release of factors that have led the cancer cells resist estrogen withdrawal and express elevated resistance to tamoxifen and ICI 182,780; co-culture experiments of macrophages with cancer cells demonstrated that TNFα together with IL-6 have led to increased activation of STAT3, NF-κB and ER*α*, thus leading to its constitutive stimulation (124).

Moreover, macrophages and CCL2 were correlated with each other and with poor survival in ER+ patients; it was also found that monocytic cells cultured with CM of tamoxifen-resistant luminal-A cells secreted elevated levels of CCL2, which then acted directly on BC cells to increase endocrine resistance *via* the PI3K/AKT/mTOR pathway (127). Along the same lines, the inflammatory chemokine CCL5 acted in autocrine manners to induce STAT3 activation, leading to tamoxifen resistance (128).

Roles for macrophages and inflammatory cytokines/ chemokines were also proposed in resistance of tumor cells to measures targeting HER2 and other receptor tyrosine kinases (129–131); there are also indications of similar roles for inflammatory cells/mediators in resistance to immunotherapy. In this specific case, the situation is even more complex because of the feedback mechanisms that dictate the equilibrium between acquired immunity and the inflammatory arm of the immune system. Here, it was demonstrated that the efficacy of immunotherapies can be repressed by myeloid cells and regulatory lymphocytes as well as by soluble pro-inflammatory mediators. It was found that MDSCs and regulatory T cells (Tregs) interfered with the beneficial effects of immunotherapies and have led to immune plasticity that was strongly connected to immune resistance (132–139). Moreover, co-targeting inflammatory pathways alongside with the use of immune checkpoint blockades has led to improved efficacy of immunotherapies (140–145).

INFLAMMATION-DRIVEN TUMOR CELL DORMANCY IN BREAST MALIGNANCY

Dormancy is another level of cancer cell plasticity that reflects the dynamic nature of disease progression, having major implications on recurrence-free rates in patients (2, 8). Dormant tumor cells are recognized by a temporary mitotic arrest, leading to a viable but non-proliferating cell state (6). Early dormancy may take place in primary tumors, but often dormancy is observed following cancer cell spreading to metastatic sites. Disseminating tumor cells that have entered dormancy can evolve and escape this state, the result being tumor relapse and disease recurrence (4, 6–8).

The mechanisms controlling entry to and escape from dormancy are currently being extensively studied. In some of the experimental systems described below, dormancy was investigated by using variant tumor cells that remained undetectable and re-emerged after a considerable long latency; in other studies, cancer cells that have entered dormancy following chemotherapy or other manipulations became proliferative and led to tumor recurrence.

It is now clear that lymphocytes such as T helper (Th) cells and CTLs establish a hostile microenvironment to the cancer cells (23, 24). Immunologic dormancy was evidenced in many tumor systems, manifesting the fact that immune mechanisms have considerable roles in determining whether the cancer cells will enter dormancy and stay quiescent, or if they will become fully equipped with the machineries that enable them to re-emerge and metastasize (17, 18, 23, 24). If this stage takes place when beneficial aspects of acquired immunity are suppressed or when the patient is not treated by tumor-limiting drugs, exit from dormancy would serve well the needs of the tumor cells and lead to recurrence and disease progression.

The activities of key immunological players like effector Th1 cells and CTLs take place alongside inflammatory processes that recently have been identified as a leading force in promoting escape from dormancy. Specifically in BC, a strong connection was revealed between inflammatory conditions and tumor cell exit from dormancy, metastasis and recurrence. This has been demonstrated under conditions like obesity-associated inflammation or inflammation induced by exposure to tobacco smoke or to lipopolysaccharide (146–148). With respect to obesity, it was found by deGraffenried and colleagues that disease recurrence was significantly reduced in ERα+ obese

patients upon use of nonsteroidal anti-inflammatory drugs (NSAIDs), which are potent inhibitors of inflammation (148).

Links were also recently made between surgery/wound healing and enhanced emergence from dormancy (149–155). This path was then connected to inflammation in BC, for example by a study addressing surgery- and chemotherapy-induced dormancy (156). This research has demonstrated that administration of ketorolac—which is an analgesic with NSAID activities targeting COX-1 and COX-2—reduced awakening from dormancy and tumor recurrence in a Lewis lung cancer (LCC) model, and that the dormancy process was mediated by COX-1 (156). Connecting these findings to BC is the fact that similar to LCC, pre-operative ketorolac administration prolonged animal survival after mastectomy in a TNBC model system (156). Furthermore, intra-operative administration of ketorolac to BC patients significantly prolonged disease-free survival (155).

Within the scope of the close connections between inflammation and dormancy, the following observations were made (summarized in **Figure 1** and **Table 3**).

Inflammation-Associated Myeloid Cells Regulating Tumor Cell Dormancy

The roles of macrophages in regulating dormancy were demonstrated in a recent study addressing wounds formed in an immunogenic model of BC (157). In this research it was found that BC cells devoid of metastatic capabilities gained the ability to disseminate and grow in remote organs after surgery. Moreover, surgical wounding has induced a systemic inflammatory response and accordingly, administration of the NSAID meloxicam, starting prior to surgery, had an inhibitory impact on tumor growth (157). The inflammatory reaction was manifested by elevated levels of circulating myeloid cells (monocytes and neutrophils), and elevated expression of inflammatory mediators, including the major monocyte chemoattractant CCL2; CCL2 down-regulation has led to partial reduction in tumor outgrowth (157).

These findings emphasize the roles of myeloid cells in dormancy control in BC. Here, it is interesting to note that breast tumors were found to be enriched with a M2-related macrophage subset that was localized in proximity to blood vessels in primary tumors and bone metastases of BC patients (158). Animal studies demonstrated that these macrophages expressed vascular endothelial growth factor A (VEGFA) and that following chemotherapy, tumors lacking VEGFA+ macrophages recurred in slower kinetics than tumors containing VEGFA+ macrophages (158).

As mentioned above, COX-1 and COX-2 were proposed as pro-inflammatory mediators that enhanced awakening tumor cells from dormancy (148, 156). The above-mentioned study by the deGraffenried group also demonstrated the close relations of COX enzymes to macrophages in context of dormancy (148). Here, sera of obese ERα+ patients induced the expression of COX-2 in macrophages, leading to greater aromatase expression by pre-adipocytes (148); then, aromatase that was released by macrophages/pre-adipocytes grown with sera derived from obese patients, induced in ERα+ breast tumor cells the activity of ERα, tumor cell migration, and proliferation (148).

Another link connecting dormancy and macrophages was made by showing the involvement of chemokines that recruit monocytes, such as CCL2 and CCL5, in such processes. Roles for CCL2 in driving forward monocyte recruitment, then leading to exit from dormancy, were discussed in the context of wound healing, as mentioned above (157). In parallel, in a research system based on HER2 down-regulation that has led to generation of residual tumors and then to tumor recurrence, CCL5 induced elevated presence of macrophages that promoted emergence from dormancy. This effect was mediated by increased presence of CCR5+ collagen-depositing macrophages in residual tumors and CCL5 over-expression in tumor cells has led to faster recurrence (159). Moreover, this CCL5 study (159) also has made an interesting connection between dormancy and TNFα activities, through the NF-κB pathway. It has shown that HER2 down-regulation has induced a pro-inflammatory program that included TNFα, which through activation of the IKK-NF-κB pathway has given rise to chemokine induction; the chemokines included CCL5 that mediated the increased abundance of CCR5+ macrophages, which contributed to escape form dormancy (159).

Pro-Inflammatory Cytokines Regulating Tumor Cell Dormancy

The above studies provided evidence to roles of macrophage-associated cytokines and chemokines in controlling cancer cell dormancy. Alongside with TNFα that was reported above, other strong pro-inflammatory cytokines were found to regulate dormancy, for example by increasing the proliferation of dormant cancer cells at a bone-like microenvironment. In a study addressing not only TNFα but also IL-1β, the two cytokines were shown to increase tumor cell proliferation in a metastasis-suppressed model of a TNBC cell line and in combination with IL-6 and CXCL8 also of luminal-A cells (160). This study has demonstrated that the NSAID indomethacin and a prostaglandin E2 (PGE2) antagonist inhibited the pro-tumor effects of the cytokines (160), thus joining other reports that provided evidence to major roles for COX enzymes and PGE2 in promoting exit from dormancy [*e.g.*, (148, 156)].

In parallel, it was found that IL-6 supported the growth of luminal-A BC cells in "dormant colonies", but lowered the proliferation of the same cells in "growing colonies" (161). These findings suggest that IL-6 may potentially have opposing roles in control of tumor cell growth and dormancy, depending on intrinsic properties of the cancer cells.

It is interesting to note that another member of the IL-6 family, leukemia inhibitory factor (LIF) was found to maintain the dormancy state in breast tumor cells (162), being in line with previous studies suggesting that LIF receptor (LIFR) is a tumor suppressor gene [*e.g.* (163)]. Low mRNA levels of LIFR and its downstream signal transducer STAT3 were significantly associated with bone metastasis and poor prognosis, respectively, in BC (162). This observation was followed up by

in vitro tests that linked LIFR down-regulation to increased migration and invasion abilities of luminal-A tumor cells; moreover, LIFR knockdown has led to increased proliferation of the tumor cells and elevated osteoclastogenesis/bone destruction in mice, in contrast to WT cells that remained in a dormant phenotype (162). Of note is the fact that low metastatic cells that migrated to the bone niche and stayed in a dormant state were sensitive to the effect of LIF : LIFR on the activation of STAT3, while TNBC cells that normally did not enter dormancy did not respond to LIF stimulation (162).

Inflammatory Chemokines Regulating Tumor Cell Dormancy

Some of the studies mentioned above addressed also CXCL8, demonstrating that it could promote escape from dormancy on one hand (160, 161), but it reduced the proliferation of breast tumor cells in "growing colonies" (161). Here, it is important to indicate that in contrast to the above-mentioned findings on CXCL8-mediated down-regulation of BC cells at growing phase (161), CXCL8 and other members of the ELR+ CXC sub-group of chemokines are generally and largely considered as key promoters of cell proliferation and viability in BC [reviewed in (50)]; thus, it is assumed that such chemokines would often enhance re-emergence from dormancy.

Supporting this view is a recent study addressing the hepatic niche in which BC cells often colonize (164). This report has demonstrated that CXCL8 was released by hepatic stellate cells and has increased TNBC cell proliferation through its receptor CXCR2 and ERK signaling activation; this pathway was relevant to dormancy because CXCL8 contributed to emergence from doxorubicin-induced dormancy in an *ex vivo* 3D liver micro-physiological system (164). In another report, the ELR+ CXC chemokine CXCL5 was shown to up-regulate BC cell proliferation in an *ex vivo* tumor-bone co-culture system that assessed the switch from dormancy to colonization through stimulation of tumor cell growth (165). In that investigation, CXCL5 expression levels were elevated in co-cultures using bones derived from cancer-bearing mice compared to bones from healthy mice; it was also found that CXCL5 increased murine breast tumor cell proliferation under quiescent conditions and that the process was mediated through CXCR2, whereas factors derived from bones of healthy mice induced cancer cell quiescence (165).

FINAL CONSIDERATIONS AND PERSPECTIVES

Extensive research has led scientists and physicians to realize that cancer cell plasticity puts its marks on tumor fate and dramatically influences disease course in malignancy. Tumor cell remodeling stands in the basis of phenotypic and functional heterogeneity and leads to constant changes in tumor characteristics, thus having cardinal clinical implications (4, 5, 9, 10).

In this review, addressing breast cancer as a representative model system, we have demonstrated that pro-inflammatory constituents enhance cancer cell plasticity by increasing stemness/EMT, therapy resistance and exit from dormancy. These three remodeling forms can be driven and up-regulated by similar pro-inflammatory elements: inflammation-associated myeloid cells, pro-inflammatory cytokines and inflammatory chemokines, as indicated in the papers summarized in this review and summarized in **Figure 1** and **Tables 1**–**3**. Moreover, the canonical transcription factors that mediate the functions of some of the mediators, namely NF-κB and STAT3, were generally implicated in generating tumor cell plasticity or maintaining it at different stages of the malignancy cascade (35, 39, 40, 115).

In this manuscript, we have portrayed an interactive remodeling scenario, in which three forms of plasticity are connected, with inflammation being a common thread that links them all. These observations set inflammation as a potential target in cancer therapy. To give an example, one therapeutic approach could be to use inflammation to trigger the awakening of tumor cells from dormancy, but to do so in a well-controlled manner that allows "hitting" the cancer cells when they enter a proliferating step, *e.g.*, by chemotherapy. However, not only that "reviving" the tumor is a risky step, but also one should take into consideration the possibility that inflammatory processes may halt mechanisms of immune surveillance that could have been beneficial in fighting the re-arising tumor.

The opposite approach could take advantage of the fact that inflammation forms a regulatory hub in promoting tumor cell plasticity, setting inflammation as a prime target for therapy whose inhibition may reduce stemness/EMT, therapy resistance and dormancy, all at the same time. Indeed, as inflammation seems to be a common denominator that strongly connects various aspects of tumor cell remodeling, inhibition of inflammatory elements may reduce a number of cell remodeling processes simultaneously.

In this context, measures that combine efforts to reduce inflammation and in parallel strengthen anti-tumor immune activities (*e.g.*, immunotherapies) (as exemplified in (140–145)), may have an even greater benefit in the clinic. This approach, that tilts the immune balance by down-regulating inflammation and increasing protective immunity, is practically feasible. For example, in the clinic, TNFα inhibitors are used with relatively high success in therapy of autoimmune diseases and inflammatory disorders, and therapies directed to IL-6 are used in rheumatoid arthritis (166–168). In parallel to the potential use of TNFα and IL-6 inhibitors, one can also consider reducing the recruitment of monocytes to primary tumors and metastases, *e.g.*, by inhibiting the activities of chemokines and their receptors, as well as of other cytokines that support monocyte migration and macrophages activities, like CSF-1 (49, 52, 143, 169). These measures can be used together with immunotherapies that have been already introduced to patients in different types of malignancies, including current clinical trials in TNBC [*e.g.* of antibodies directed to PD-L1 (170)].

Obviously, it is not an easy task to tune the equilibrium in favor of anti-tumor activities and more efficient immune surveillance; moreover, one should consider the possibility that specific immune/inflammatory mediators or transcription factors may have opposing roles depending on intrinsic and extrinsic signals. One example to such a problematic scenario

was described above, when STAT3 was found to have key roles in promoting cancer cell remodeling, *e.g.*, following IL-6 activation, but in response to LIF activation kept dormant BC cells in check (42, 115, 171).

These aspects emphasize the need to carefully identify the inflammatory mechanisms regulating tumor cell plasticity. Thus, to successfully implement a combined tactic of inhibiting inflammation while promoting protective immunity, a detailed and accurate analysis of the elements involved in driving and regulating tumor cell remodeling should be performed in each and every cancer type and subtype.

AUTHOR CONTRIBUTIONS

TB, LR-A, and HB-Y contributed to literature search, manuscript writing and editing. HB-Y also assisted in figure preparation. AB-B was responsible for the entire setup and structure design of the manuscript, and participated in all other aspects of article preparation including writing, editing, and figure design. All authors contributed to the article and approved the submitted version.

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REVIEW
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Could Extracellular Vesicles Contribute to Generation or Awakening of "Sleepy" Metastatic Niches?

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Pre-metastatic niches provide favorable conditions for tumor cells to disseminate, home to and grow in otherwise unfamiliar and distal microenvironments. Tumor-derived extracellular vesicles are now recognized as carriers of key messengers secreted by primary tumors, signals that induce the formation of pre-metastatic niches. Recent evidence suggests that tumor cells can disseminate from the very earliest stages of primary tumor development. However, once they reach distal sites, tumor cells can persist in a dormant state for long periods of time until their growth is reactivated and they produce metastatic lesions. In this new scenario, the question arises as to whether extracellular vesicles could influence the formation of these metastatic niches with dormant tumor cells? (here defined as "sleepy niches"). If so, what are the molecular mechanisms involved? In this perspective-review article, we discuss the possible influence of extracellular vesicles in early metastatic dissemination and whether they might play a role in tumor cell dormancy. In addition, we comment whether extracellular vesicle-mediated signals may be involved in tumor cell awakening, considering the possibility that extracellular vesicles might serve as biomarkers to detect early metastasis and/or minimal residual disease (MRD) monitoring.

Keywords: extracellular vesicle, exosome, dormancy, metastasis, disseminated tumor cells

INTRODUCTION

There is evidence suggesting that tumor cells disseminate from the very beginning of primary tumor formation (Hosseini et al., 2016). Disseminated tumor cells (DTCs) in circulation eventually reach specific distal sites where these metastatic cells become quiescent (Goddard et al., 2018). This phenomenon, known as tumor dormancy, could be maintained for several years before these cells reactivate to generate secondary lesions, explaining how metastasis can appear in cancer patients with no evidence of disease after successful treatments, even after complete resection of the primary tumors. The knowledge that dissemination happens early in tumor development has challenged traditional models of metastatic progression, representing a change in the paradigm that metastatic cells only appear late on in tumor progression (Harper et al., 2016; Suhail et al., 2019). Regardless of the mechanisms involved, metastasis is considered an inefficient process (Luzzi et al., 1998) as most DTCs that leave the primary tumor die through apoptosis or immune clearance

along their journey (Mehlen and Puisieux, 2006). As such, only a few of them successfully reach distal organs, extravasate and once there, survive in a quiescent state. The specific mechanisms underlying tumor latency in dormant niches is beginning to be defined, suggesting a key role for the microenvironment in these niches (Bissell and Hines, 2011). The balance between DTC dormancy and cell awakening is conditioned by signals either from tumor cells or from stromal components within the surrounding area, including signals from the extracellular matrix (ECM), the vasculature and the immune system (Ghajar, 2015; Aguirre-Ghiso, 2018). Evidence has accumulated over recent years that the niche surrounding the microvasculature (e.g., the perivascular niche -PVN) orchestrates DTC dormancy, principally responsible for cell survival and growth arrest (Ghajar, 2015). There is also strong evidence that certain ECM proteins promote cell dormancy, such as the thrombospondin-1 (THBS1) of the microvascular endothelium in breast cancer (Ghajar et al., 2013) and osteopontin within the bone marrow in leukemia (Boyerinas et al., 2013). Some ECM factors like fibronectin induce entry into a dormant cell phenotype, which is dependent on soluble factors like transforming growth factor-β (TGFβ), Barney et al., 2020) suggesting that ECM and soluble factors may join forces to regulate DTC dormancy. However, other works also showed that fibronectin reawakes dormant tumor cells (Barkan et al., 2008; Eyles et al., 2010), supporting that depending on the model used, fibronectin may have a differential role regulating dormancy/awakening.

Interestingly, extracellular vesicles (EVs) have emerged as important messengers in cell-cell communication (Tkach and Thery, 2016), although the contribution of EVs to tumor cell dormancy is still poorly understood. As such, we will discuss here the potential role of EVs in the communication between tumor and stromal cells, and their influence on tumor cell dormancy and awakening in metastasis.

MICROENVIRONMENTAL REGULATION OF DTC DORMANCY

Metastatic lesions preferentially develop in specific anatomical locations (Paget, 1989), suggesting that a combination of intrinsic and extrinsic factors dictates the success of DTC colonization (Bragado et al., 2012). The characteristics of the dormant niche include the changes to the microenvironment that favor metastatic cell survival (e.g., stem cell properties, immune, and endothelial cell changes) (Goddard et al., 2018), which together with the ECM and hypoxic microenvironments (Fluegen et al., 2017) modulate the characteristics of the dormant niche.

Stem Cell or Dormant Niches

The ability of different organs to support DTC growth can be classified as dormancy-permissive or dormancy-restrictive (Bragado et al., 2012). It has been proposed that stem cell niches are specialized microenvironments that could support the survival of DTCs (Sosa et al., 2014; Ghajar, 2015; Hen and Barkan, 2020) and that they share mechanisms for cell recruitment, for example, attracting DTCs expressing CXCR4 through the secretion of G-CSF or CXCL12 in the case of prostate cancer (Shiozawa et al., 2011a). Once DTCs reach hematopoietic niches, several locally secreted factors induce DTC dormancy, such as growth arrest-specific protein 6 (GAS6) (Shiozawa et al., 2011b), bone morphogenetic protein 7 (BMP7) or BMP4 in the case of lung niches (Kobayashi et al., 2011; Gao et al., 2012), and TGFβ2 (Bragado et al., 2013).

Alternatively, dormant tumor cells acquire stem cell-like properties and they overexpress pluripotential and self-renewal genes in dormant niches (Calabrese et al., 2007; Sosa et al., 2015; Malladi et al., 2016). For example, the Notch2 pathway induces stem phenotypes resembling hematopoietic stem cells (HSCs), which mediates breast cancer cell dormancy in the endosteal niche of the bone (Capulli et al., 2019). MTOR signaling and a higher proportion of p38-MAPK relative to ERK activation is also necessary to maintain the quiescence of both DTCs and cancer stem cells (CSCs) (Aguirre-Ghiso et al., 2001; Hen and Barkan, 2020). In a model of colorectal cancer (CRC), a sub-population of quiescent cells expressing ZEB2 display stemness and mesenchymal properties, and they have been associated with chemoresistance (Francescangeli et al., 2020). FBXW7 is also expressed strongly in several populations of stem cells, sustaining lung adenocarcinoma and breast cancer dormancy by blocking entry into the cell cycle (Zhang et al., 2019). Thus, in several tumor sub-types dormant tumor cells are commonly referred to as slow-cycling CSCs that combine quiescent properties with tumor initiating and chemoresistant properties, which favor later relapse and for the formation of metastases [(De Angelis et al., 2019a,b) and references therein].

Immune Cell-Induced DTC Dormancy

The adaptive immune system contributes directly to tumor cell dormancy in different ways (Feuerer et al., 2001; MacKie et al., 2003; Ross, 2007; Schreiber et al., 2011). It has been proposed that $CD8^+$ T cells induce tumor cell dormancy and indeed, spontaneous metastases can be controlled and maintained in a dormant state by the wild-type (WT) immune system in mouse models, with no application of any anti-cancer treatment (Romero et al., 2014a). Interestingly, the recurrence of metastasis in mice depleted in T cells occurs with a much shorter latencies than in models with functional T cells (Farrar et al., 1999; Koebel et al., 2007; Eyles et al., 2010; Romero et al., 2014a; Farhood et al., 2019). A key issue in this process is whether the dormant metastases are in a quiescent state or a state of equilibrium between tumor cell proliferation and cell death, an issue not yet fully clarified. Regardless of the mechanism involved, data suggest that the interaction between MHC-I molecules on the cancer cell surface and T-cell receptors may play an important role in tumor cell dormancy (Koebel et al., 2007; Romero et al., 2014a,b). Although less significantly, $CD4^+$ T cells are also involved in DTC dormancy (Romero et al., 2014a,b; Borst et al., 2018) as they induce tumor cell dormancy and cell cycle arrest through TNFR1 and IFN-γ signaling in pancreatic cancer (Muller-Hermelink et al., 2008). Additionally, $CD4^+$ T cells secrete inhibitors of angiogenesis (e.g., CXCL9 and CXCL10) which can indirectly contribute to tumor dormancy

by stabilizing the endothelium (Muller-Hermelink et al., 2008; Pardee et al., 2010).

The innate immune system may also be involved in tumor cell dormancy, as is the case of natural killer (NK) cells (Wu et al., 2013; Malladi et al., 2016), the cytotoxic capacity of which is mediated by perforin secretion and is very relevant in this process (Brodbeck et al., 2014). Interestingly, NK cells can also provide a variety of cytokines (e.g., CXCL10) that enhance the aforementioned ability of CD8 and CD4 lymphocytes to induce dormancy in a model of acute myeloid leukemia (AML)(Saudemont et al., 2005), however, this study shows only correlation of NK ligand expression with dormancy markers in dormant tumor masses without showing function of NK. Additionally, a recent study showed co-localization of dormancy markers (e.g., H2BK or PDGFB) with ligands of the NK group 2 member D receptor (MICA, MICB, or ULBP1 and 2) in patients with brain metastasis from lung and breast cancers, suggesting that these mechanisms may co-operate in maintaining metastatic dormancy (Fluh et al., 2020).

Other innate immune cells like macrophages are responsible for the survival of dormant breast cancer cells just after their extravasation into the lung. Aberrant expression of vascular cell adhesion molecule-1 (VCAM-1) was seen to favor the interaction of extravasated breast cancer cells with metastasis associated macrophages, an interaction that activates Akt via Ezrin, and that eventually offers metastatic cells protection against cytokine induced apoptosis (Chen et al., 2011). However, the role of this cell type in tumor dormancy remains controversial, since their polarization to a M1 or M2 phenotype seems to condition their effects on tumor cells (Yang et al., 2016).

Alternatively, dormant DTCs acquire characteristics that favor immune cell evasion and cell survival (Racila et al., 1995; Farrar et al., 1999; Muller-Hermelink et al., 2008; Teng et al., 2012; Goddard et al., 2018; Liu et al., 2018). Thus, tumor antigen expression by dormant DTCs is dampened or the expression of checkpoint inhibitors or immunosuppressive molecules induced (e.g., PD-L1, CTLA-4, CD80, CD39, and CCR4), which results in immune cell evasion (Saudemont and Quesnel, 2004; Linde et al., 2016; Malladi et al., 2016; Flores-Guzman et al., 2020). Furthermore, dormant cells decrease their MHC I levels as another means to evade T cell responses (Pantel et al., 1991; Pommier et al., 2018; Romero et al., 2018). This is a specific requirement for $CD8^+$ T cell immune evasion, since MHC-I remains intact in tumor cells implanted into immunodeficient or T cell immunodepleted mice (Garcia-Lora et al., 2001; Romero et al., 2018). Interestingly, in both a pancreatic ductal adenocarcinoma mouse model and patients with liver metastasis, single DTCs do not express MHC-I or cytokeratin 19 (CK19). The downmodulation of MHC-I in dormant DTCs seems to be mediated by ER stress-dependent activation of the unfolded protein response (UPR) (Pommier et al., 2018). However, other studies suggest that MHC-I is enhanced on the surface of dormant tumor cells, generating long-term memory in $CD8^+$ T cells (Perez et al., 1990; Mahnke et al., 2005; Romero et al., 2014b). Moreover, MHC-I expression on dormant cells could itself guarantee a quiescent state, since MHC-I molecules can have a direct tumor suppressor role and arrest tumor progression (Garrido et al., 2012). Together, these data suggest the possibility that both MHC-I positive and negative cells are present at dormant niches, maintained in an equilibrium that allows dormant cells to adapt to the different microenvironmental scenarios.

The presence of regulatory T cells (Tregs) in immunogenic niches might also favor the dormant niche. Interestingly, bone marrow is one of the best characterized dormant niches and it represents an important reservoir for Tregs (Zou et al., 2004). Since memory T cells could be correlated with the presence of DTCs, for example in bone metastasis of advanced breast cancer patients (Feuerer et al., 2001; Mahnke et al., 2005), it is tempting to suggest that Tregs could block the complete activation and functionality of resident memory T cells. Likewise, Treg populations increase in dormant tumors in a mouse B cell lymphoma model (BitMansour et al., 2016). Together, these data suggest that the success of the metastatic outcome depends on interactions between the immune system and DTCs, influencing the equilibrium between tumor cell proliferation and cell death (Chen et al., 2011).

Endothelial Cell-Induced Dormancy

DTCs from various tumor types interact with the lung, bone marrow and brain vasculature at distant metastatic sites (Chambers et al., 2002; Kienast et al., 2010; Ghajar et al., 2013; Price et al., 2016; Bridgeman et al., 2017; Yadav et al., 2018). The attachment of DTCs to endothelial cells favors their survival, differentiation and the growth arrest of DTCs at dormant niches (Yadav et al., 2018). Several authors suggested that a stable endothelium may favor the survival of dormant tumors and reduce tumor growth, whereas neovascularization is associated with tumor growth (Gimbrone et al., 1972; Hawighorst et al., 2002; Folkman and Kalluri, 2004). This phenomenon could also be applied to the dormancy of micrometastases, which remain in this sleepy state when neo-angiogenesis is suppressed (Holmgren et al., 1995). Indeed, in a model of human ovarian cancer dormancy, anti-angiogenic genes are the genes most frequently affected, with enhanced TIMP3, TSP1, Ang1, and CDH1 expression as part of in dormant signature that is dampened upon tumor relapse and recurrence (Lyu et al., 2013). Thus, these data suggest that vascular niches play key roles in the fate of dormant cells. In 2013, it was demonstrated for the first time that the vascular endothelium induces tumor cell quiescence in an *in vivo* model of breast cancer dissemination (Ghajar et al., 2013), the THBS1 produced by endothelial cells promotes tumor cell dormancy. The role of THBS1 in the maintenance of tumor dormancy of breast invasive ductal carcinoma has been corroborated, pointing to tryptophan as key source for the production of THBS1 by endothelial cells (Lopes-Bastos et al., 2017). Moreover, BMP-4 signaling associated with the induction of dormancy, also augments THBS1 expression in the lung endothelium (Gao et al., 2012; Lee et al., 2014). In addition to THBS1, downregulation of VCAM1 and the lysophosphatidic acid receptor (EDG2) is also required to guarantee the dormant state of DTCs (Lu et al., 2011; Marshall et al., 2012). Interestingly,

angiogenic dormancy may also contribute to tumor dormancy due to the lack of nutrients in poorly vascularized niches (Senft and Ronai, 2016; Natale and Bocci, 2018). This could control the balance between cancer cell proliferation and apoptosis. Angiostatin, a circulating inhibitor of angiogenesis, could be one of the molecules responsible for this phenomenon (O'Reilly et al., 1994; Cao et al., 1998).

Although the PVN seems to be a favorable niche for DTC dormancy, several microenvironmental cues regulate this phenomenon in specific organs. In the brain, vascular co-option of tumor cells adhered to the abluminal surface of the vasculature is strictly necessary for DTC survival (Kienast et al., 2010; Zhang et al., 2020). Moreover, astrocyte and microglial responses that promote local changes in the tumor microenvironment favor or restrict breast tumor progression (He et al., 2006; Kienast et al., 2010; Lorger and Felding-Habermann, 2010). Arrested and/or extravasated tumor cells could also activate both astrocytes and microglia in their vicinity. Astrocyte activation, identified by the up-regulation of GFAP and Nestin, leads to the expression of matrix metallopeptidase 9 (MMP-9). Since this is one of the first events in the metastatic colonization of the brain, reactive astrocyte-dependent MMP-9 secretion might create a niche that supports brain metastatic lesions (Lorger and Felding-Habermann, 2010). Alternatively, the stellate or amoeboid activated microglia cells (high F4/80 expression) could secrete multiple soluble factors that modulate both proliferative and anti-proliferative tumor responses (He et al., 2006; Lorger and Felding-Habermann, 2010). Similarly, pancreatic ductal adenocarcinoma cells use hepatic stellate cells of the sinusoidal capillaries to establish their dormancy in the liver (Lenk et al., 2017; Fabian et al., 2019). It was proposed that hepatic stellate cells induce a dormant phenotype of pancreatic ductal tumor cells through the secretion of IL-8 (Lenk et al., 2017). Indeed, hepatic stellate cells-mediated tumor quiescence is thought to also be regulated by changes in oxidative metabolism (i.e., Succinate Dehydrogenase subunit B -SDHB- expression), affecting cell growth and the stem properties of liver metastasis from pancreatic tumors (Fabian et al., 2019). In the bone, dormant cells are usually found in E-Selectin and SDF-1 rich perisinusoidal vascular areas (Price et al., 2016), which favor their entry and establishment in the bone, respectively. The close proximity of skeletal vascular networks and hepatic niches favors a microenvironment rich in ECM proteins, secreted factors like THBS1, Stem cell factor (SCF-1) or the chemokine CXCL12 that sustain tumor dormancy (Kusumbe, 2016).

In summary, the success of metastasis relies on both the microenvironment of the metastatic dormant niches and the cancer cells involved (Pencovich et al., 2013; Ghajar, 2015; Wang et al., 2015; Carlson et al., 2019). Different approaches have focused on targeting the microenvironment of metastatic dormant niches to maintain the DTCs in a quiescent state or alternatively, promoting their awakening to sensitize them to therapies (Carlson et al., 2019). Nevertheless, this scenario is very complex and unlikely to be used in the clinic at present, at least until further knowledge and a better understanding of the PVN and DTC interactions can be successfully employed to promote "eternal DTC sleepiness."

INFLUENCE OF EXTRACELLULAR VESICLES IN TUMOR CELL DORMANCY

One of the main questions regarding the regulation of DTC dormancy concerns the mechanisms involved in the communication between stromal cells in the niche and the DTCs. EVs are thought to be important participants in intercellular communication, yet their role in the communication between DTCs and their niches is still unclear. EVs can be classified based on their origin and size. A recent classification based on size divided them into large (lEVs) and small EVs (sEVs) (Witwer and Théry, 2019), whereby microvesicles (200 nm–1 μm), apoptotic bodies (1–5 μm) and oncosomes (1–10 μm) can be considered lEVs, yet smaller vesicles like exosomes and exomeres are considered as sEVs (Witwer and Théry, 2019). EVs are a heterogeneous population of vesicles that are secreted depending on the biological context (Di Vizio et al., 2012; Zhang H. et al., 2018; Ren et al., 2019). Regardless of their origin, EVs transport proteins, lipids, and nucleic acids (both RNA and DNA) that are representative of the cell of origin (Choi et al., 2013; Raposo and Stoorvogel, 2013). Once EVs reach their target cell they can transfer their cargo horizontally, modulating physiological and pathological processes (Colombo et al., 2014). Some examples of their broad functions include their role in the cross-talk of immune cells (Thery et al., 2009), in the regulation of coagulation (Tripisciano et al., 2017) or in the formation of the pre-metastatic niche (Peinado et al., 2017). Thus, although there are only a few studies suggesting a role of exosomes in the regulation of tumor cell dormancy, there is an increase in the number of studies showing that EVs are involved in processes like vascular leakiness, extracellular remodeling and regulation of the immune system (Becker et al., 2016). Since these are processes crucial to the establishment of dormant niches, it is plausible that secreted EVs play a role regulating DTC dormancy.

Extracellular Vesicles in the Cross-Talk Between Stem Niches and Dormant Cells

Homing and survival at distal sites is the most rate-limiting step in the metastatic cascade (Valastyan and Weinberg, 2011). Several studies have highlighted how stromal cell-secreted EVs influence the behavior of tumor cells in specific niches, mainly through the transfer of miRNAs (**Figure 1**). For example, stromal cell-secreted EVs could be differentiated from tumor-secreted EVs based on their size, their protein and miRNA content, supporting the idea of effective bi-directional cross-talk between stromal and tumor cells in the niche that sustains tumor cell colonization and dormancy (Dioufa et al., 2017). In bone marrow niches, MDA-MB-231 and T47D breast cancer tumor cells prime mesenchymal stem cells (MSCs) to release EVs containing distinct miRNAs, such as miR-222/223, which the authors propose to induce quiescence of a subset of cancer cells and confers drug resistance (Bliss et al., 2016). Of note, treatment induced only a small fraction of tumor cells into G1-G0 phase while naïve MSC-derived EVs actually induced the majority of MDA-MB-231 cancer cells into cycling. In another study, the treatment of

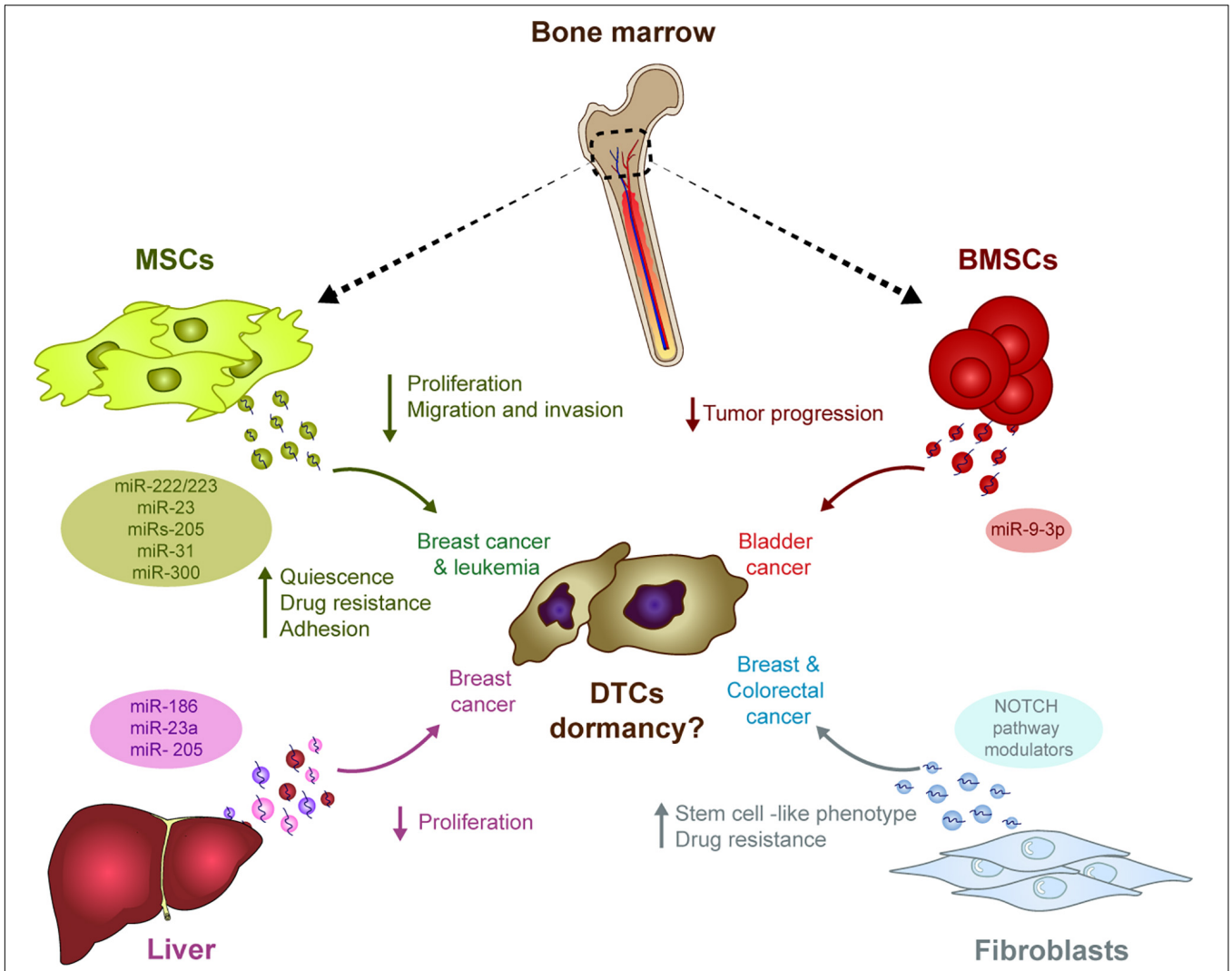

FIGURE 1 | The influence of stromal-derived EVs in tumor cell dormancy. EVs generated in different stromal cells may have the ability to induce DTC dormancy in diverse tumor types. The transport of miRNA in these EVs outstand as one of the main mechanism potentially involved in this process: In the bone marrow, MSCs-derived EVs contain different miRNAs capable of modulating several pro-dormant features (e.g., quiescence, reduced proliferation, etc.) in breast cancer cells, whereas miRNAs in BMSCs-derived EVs suppress progression of bladder cancer. In the liver, several miRNAs in hepatic niche-derived EVs reduced breast cancer cell proliferation. Finally, miRNAs in EVs derived from fibroblasts induce a stem cell like phenotype and resistance to therapy in breast and colorectal cancer cells.

BM2 cells (bone metastatic human breast cancer cells derived from a MDA-MB-231 parental cell line) with EVs from MSCs suppressed cell proliferation, inhibits invasion and dampens their sensitivity to docetaxel. Authors found that miR-23b was responsible of this effect by inducing dormant phenotypes through the suppression of MARKS expression (which encodes a myristoylated alanine-rich C kinase substrate) (Ono et al., 2014). In this work, however, the relevance of these findings were mainly verified *in vitro*. The *in vivo* experiments lack from verification of cell death after engraftment of metastatic breast cancer cells treated with MSC-derived exosomes. Further *in vivo* data and cell death analysis is needed to understand the relevance of these findings. Controversially, MSC EVs were only seen to suppress the metastatic potential of parental MDA-MB-231 cells but not that of metastatic MDA-231 organ tropic models (Vallabhaneni et al., 2017). In this work, authors propose that MSC EVs induced dormancy through a mechanism dependent on miRs-205 and 31, suppressing the expression of the UBE2N/Ubc13 gene that is correlated with reduced proliferation, and suppressed migration and invasion of breast cancer cells *in vitro* (Vallabhaneni et al., 2017). Nevertheless, the effect on cell death was not measured in MDA-231 cells treated with naïve exosomes from MSC and detection of remaining DTCs was not provided to analyze if DTCs were present and remained dormant in distant organs.

In addition to MDA-MB-231-derived models, treatment with hMSC EVs also reduced the proliferation and migration of MCF7 breast cancer cells, while enhancing the adhesion (Casson et al., 2018) or suppressing the progression of bladder cancer cells through ESM1 downregulation by bone marrow stem cell

(BMSC)-derived exosomal miR-9-3p (Cai et al., 2019). Apart from this suggested role regulating dormancy in solid tumors, miRNAs contained in MSCs EVs have also been described to play a role in hematological malignancies. In this context, miR300 was found to contribute to the persistence of drug-resistant quiescent leukemic stem cells in chronic myelogenous leukemia. Upon miR300 upregulation in malignant cells by different sources, including EVs transference from bone marrow MSCs, leukemic cell proliferation was found to be impaired (Silvestri et al., 2020).

Hepatic niche (HepN, fresh human hepatic tissue) derived exosomes contribute to breast cancer cell homing, survival and dormancy when studied in an *ex vivo* human liver microphysiological system. In this system, MDA-231 breast cancer exosomes were used to prime the hepatic cells. Later, the primed HepN-derived EVs alter the levels of several miRNAs involved in epithelial cell differentiation (miR186, miR23a, and miR205), with a concomitant reduction of cancer cell proliferation (Dioufa et al., 2017). Exosomes from fibroblasts can also induce stem cell like features and resistance to therapy via the Notch pathway in several cancer models (e.g., breast and colorectal cancers), two intrinsic characteristics of dormant cells (Boelens et al., 2014; Hu et al., 2019).

Taking all these data together, if stromal-derived EVs influence tumor cell dormancy is a matter of current debate. We have reviewed the information in the literature and draw any conclusion would be premature at this time. Works showed that stromal cell-derived EVs may affect some characteristics of tumor cells such as proliferation, further data is needed to support their role in DTC dormacy (**Figure 1**). Several limitations in the interpretation must be borne in mind: (1) most of the studies analyzed cell proliferation rather than dormancy and some of the effects were only demonstrated *in vitro*; (2) the dose of exosomes and the models used (e.g., MSCs) are not always clearly defined; and (3) these studies are restricted to a limited number of cell models and immunodeficient mouse models, which limits the interpretation of the results. Further data *in vivo* and in immunocompetent models will help to understand if stroma-secreted EVs are involved in regulating tumor cell dormancy.

Similarly, while endothelial cells play a central role regulating tumor cell stemness, there is very little data suggesting that EVs secreted by endothelial cells are involved in tumor cell dormancy. In fact, endothelial cell-derived EVs have been implicated in tumor cell awakening rather than dormancy (see below, section "Similarities Between the Pre-Metastatic and the Awakening Niches"). As an exception to this statement, it was recently described that endothelial cells in the bone marrow supply miR-126 to chronic myelogenous leukemia stem cells to support quiescence, which correlates with poor prognosis. MiR-126 is highly expressed in normal HSCs and hematopoietic progenitor cells and restrains cell-cycle progression during hematopoiesis. However, in leukemic cells, miR-126 is down-regulated through a BCR-ABL-dependent mechanism. EVs from the surrounding bone marrow endothelial cells has been described as the main source to compensate the downregulation, this way allowing the reinduction of quiescence in a percentage of the leukemic cells, contributing to self-renewal, engraftment and perpetuation of the disease (Zhang B. et al., 2018). Nevertheless, since studies into tumor cell dormancy normally require an *in vivo* setting, analyzing the involvement of EVs in this process is very challenging. The development of models to properly demonstrate the influence of endothelial cell- and immune cell-derived EVs will be necessary to demonstrate their relevance, and their role *in vivo* must be compared with that of soluble and intrinsic factors.

Extracellular Vesicles in the Regulation of Immune Dormancy

Very little is known about the role of EVs derived from immune cells in directly controlling tumor cell dormancy. There is no data in the literature linking T Cell or NK-cell derived EVs to this phenomenon. EVs derived from specific sub-populations of immune cells could have different outcomes at dormant niches. Exosomes derived from M2 macrophages reduce proliferation and limit cell cycle progression in bladder cancer models, while M1 macrophages appear to be involved in tumor cell awakening (Walker et al., 2019), as will be discussed below (see section "The Contribution of Secreted Extracellular Vesicles to Tumor Cell Awakening"). Moreover, MHC-I molecules can also be transferred between tumor and immune cells in EVs (Lynch et al., 2009; Duchler et al., 2019), potentially affecting immune responses at metastatic sites.

Interestingly, tumor-secreted EVs may contribute to immunosuppression by favoring DTC survival. Several tumor types release EVs that carry PD-L1 on their surface, mostly in the form of exosomes, and these may suppress the activity of CD8 T cells and facilitate tumor growth. Indeed, PD-L1 expression in the plasma of patients with several tumor types is correlated with a worse patient outcome and weaker immune responses (Chen et al., 2018; Theodoraki et al., 2018; Yang et al., 2018; Fan et al., 2019; Kim et al., 2019; Li C. et al., 2019; Xie et al., 2019). Therefore, tumor-secreted exosomes containing PD-L1 may potentially offer resistance to immunotherapy (Xie et al., 2019). Indeed, blockade of tumor-derived exosomal PD-L1 restores global anti-tumor immunity even in models resistant to anti-PD-L1, and this blockade suppresses tumor growth in addition to that produced by anti-PD-L1 antibodies (Poggio et al., 2019). Since dormant DTCs express less tumor antigen or induce PD-L1 expression, driving immune cell evasion (Linde et al., 2016), it is likely that DTCs can secrete PD-L1^{+} EVs and reinforce immunosuppression in niches. However, there is no data yet supporting this hypothesis in a metastatic setting.

THE CONTRIBUTIONS OF SECRETED EXTRACELLULAR VESICLES TO TUMOR CELL AWAKENING

A particular challenge when considering metastasis is to understand which signals are involved in the outgrowth of quiescent DTCs (Braun et al., 2000). Multiple signals are involved in the reactivation of silent DTCs through a process known as re-awakening (Aguirre-Ghiso, 2018). This metastatic cell re-awakening leads to the generation of secondary lesions, which

in many cases constitute the cause of death of cancer patients (Chaffer and Weinberg, 2011). Although this condition has been largely documented by clinicians, the molecular mechanisms underlying this process and specially, the contribution of EVs to these events, are still to be defined. Successful metastasis requires a supportive microenvironment in which the DTCs can proliferate, otherwise these cells will remain quiescent in these niches (Luzzi et al., 1998; Shibue et al., 2012; Lambert et al., 2017).

Potential Involvement of EVs in DTC Awakening

As mentioned previously, stromal cell-derived EVs may be involved in DTC dormancy *in vitro*, whereas tumor-derived EVs may contribute to tumor progression and could be involved in tumor cell awakening (**Figure 2**). It was recently shown that neuroblastoma-derived EVs (Nakata et al., 2017) and lung cancer-derived EVs (Li et al., 2016) are captured by bone marrow-derived MSCs *in vitro*, inducing the secretion of pro-tumoral cytokines and chemokines like interleukin-6 (IL-6), IL-8/CXCL8, vascular endothelial cell growth factor (VEGF) and monocyte-chemotactic protein-1 (MCP-1) (Li et al., 2016; Nakata et al., 2017). Similarly, sEVs derived from ovarian cancer spheroids (with a cancer stem cell phenotype) modify the activity of MSCs and induce the secretion of IL-6, IL-8, and VEGF-A. Interestingly, exposure to cisplatin alters the cargo of sEVs released by ovarian cancer tumor cells, inducing a pro-tumorigenic activity of MSCs *in vitro* (Vera et al., 2019). Elsewhere, exosomes from ovarian cancer cell lines were seen to induce a myofibroblastic phenotype and activity in MSCs by activating different intracellular signaling pathways depending on the model used (Cho et al., 2011). Overall, it seems that tumor-derived EVs co-opt MSCs and re-program them to produce a pro-tumorigenic activity. The growth of these re-programmed MSCs is enhanced directly, as is the horizontal growth of fibroblasts, endothelial cells and immune cells in the tumor microenvironment, indirectly promoting the pro-tumor activity of MSCs (Whiteside, 2018). The secretion of IL-6, IL-8, and VEGF, and the activation of pro-inflammatory pathways seem to be the two canonical mechanisms by which MSCs respond to tumor cell-derived exosomes. Interestingly, glioblastoma-derived EVs also induce an increase in the secretion of VEGF and IL-6, as well as an increase in the phagocytotic capacity of macrophages, reinforcing their tumor-supportive phenotypes

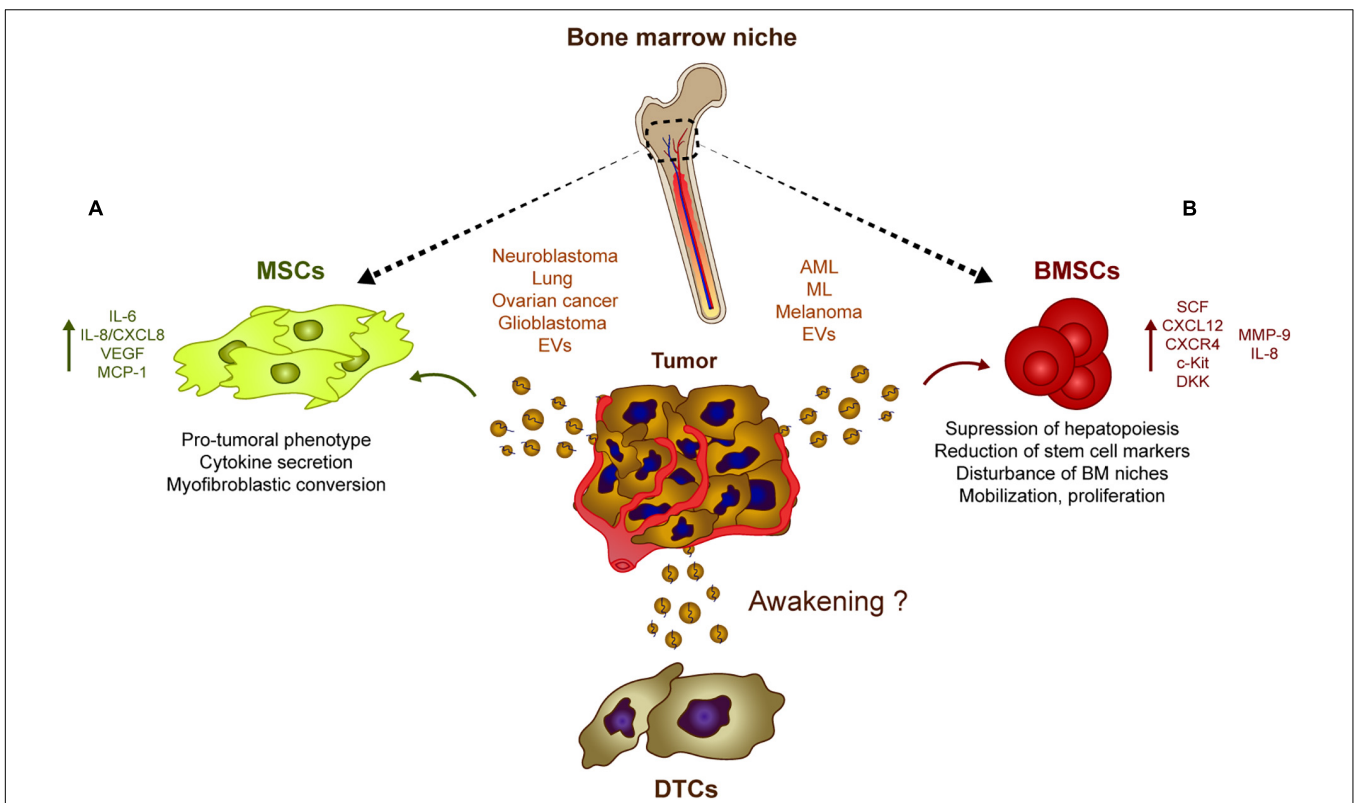

FIGURE 2 | Tumor-derived EVs influence stromal populations. Rather than inducing DTC dormancy, tumor-derived EVs could contribute to their re-awakening through different mechanisms. **(A)** Neuroblastoma, glioblastoma, lung, and ovarian cancer generate EVs capable of inducing a pro-tumoral phenotype in MSCs which usually involves a myofibroblastic conversion. These modified MSCs eventually upregulate the secretion of pro-tumoral cytokines and chemokines such as IL-8, IL6, and VEGF. **(B)** Similarly, melanoma or leukemia cells release EVs that are captured by BMSCs which induces the secretion of IL8, CXCL12 among other soluble factors. This tumor EV-mediated release of cytokines and chemokines has an effect on the bone marrow physiology suppressing hematopoiesis and disturbing several features of the bone marrow progenitors, which favor the growth of tumor cells at dormant niches.

(de Vrij et al., 2015). Analyzing the relevance and importance of EVs relative to other molecules *in vivo* will be crucial to understand if tumor-derived EVs could act as "awakeners" (**Figure 2**) as opposed to the potential role of MSC-derived EVs in "sleepy" niches (**Figure 1**).

Similarly, there is growing evidence that leukemic cells can modulate the EVs in their host bone marrow microenvironment to survive and expand. AML-derived EVs suppress normal hematopoiesis by inhibiting protein synthesis and they induce long-term hematopoietic stem cell quiescence through the internalization of miR-1246 (Abdelhamed et al., 2019) or by inducing the expression of DKK1, a suppressor of normal hematopoiesis (Kumar et al., 2018). Exosomes also regulates hematopoietic stem and progenitor cells indirectly by decreasing the SCF and CXCL12 in bone marrow-derived MSCs, or by reducing CXCR4 and c-Kit expression, triggering the ensuing suppression of hematopoietic transcription factors like c-Myb, Cebp-β, and Hoxa-9 (Huan et al., 2015; Hornick et al., 2016). In addition, amphiregulin enriched exosomes from chronic myelogenous leukemia augment the adhesion and proliferative advantage of tumor cells within the hematopoietic niche by mediating the expression of MMP-9 and IL-8 (Corrado et al., 2016). Moreover, melanoma-derived EVs are involved in the mobilization of bone marrow progenitor cells and in pre-metastatic niche formation by enhancing c-Met signaling in hematopoietic progenitors, disturbing the natural physiology of bone marrow niches (Peinado et al., 2012). These studies suggest that tumor-derived EVs affect the normal physiology of hematopoietic and mesenchymal stem cells, preceding tumor cell invasion, and helping to promote tumor cell proliferation and survival within the niches (**Figure 2**).

Similarities Between the Pre-metastatic and the Awakening Niches

It has been proposed that the primary tumor is involved in the preparation of secondary organs for the arrival and growth of tumor cells, known as the pre-metastatic niche (PMN) (Peinado et al., 2017). During the last decade, exosomes have been proposed as key players in this process (Kahlert and Kalluri, 2013; Becker et al., 2016; Lobb et al., 2017; Steinbichler et al., 2017). Interestingly, many of the modifications influenced by secreted EVs could also affect DTC awakening, such as: (1) Increasing in metastatic behavior, tumor cell survival and angiogenesis; (2) promoting ECM remodeling; (3) and favoring the recruitment of bone marrow-derived cells and local inflammation described during the generation of PMNs (Peinado et al., 2017). The similarities between both processes, raises interesting questions, such as could similar mechanisms be involved in the generation of PMNs and in DTC re-awakening? Could EVs be involved in DTC re-awakening together with other factors?

The Role of EVs in Metastasis, Tumor Cell Survival and Angiogenesis

There is evidence that endothelial cell-derived EVs influence tumor cells and for example, human brain microvascular endothelial-derived exosomes are thought to favor lung cancer tumor cell survival and resistance to apoptosis by increasing the levels of S100A16 (Xu et al., 2019). In a model of glioma, CD9-enriched EVs derived from endothelial cells also enhance glioma stem cell tumorigenesis by activating the BMX/STAT3 axis (Li D. et al., 2019). YAP1 depletion or inhibition in vascular endothelial cells increases the release of exosomes that contain the long non-coding RNA MALAT1 into the tumor microenvironment (Li et al., 2020). Exosomal transfer of MALAT1 to hepatic cells favors hepatic cell invasion and migration due to the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) signaling (Li et al., 2020). Interestingly, HUVEC release VEGF-enriched exosomes that may combat anti-angiogenic treatments, a phenomenon that favors tumor neo-vasculogenesis and tumor progression in hepatocellular carcinomas (Zeng et al., 2019). These data raise an interesting question, since endothelial cells are involved in tumor cell dormancy, could their secreted EVs take part in processes related to tumor cell awakening? One potential explanation is that while the physical interactions between stable endothelial and tumor cells are crucial in early tumor cell homing and to induce DTC dormancy, as the disease evolves, sprouting of endothelial cells could promote the secretion of EVs involved in DTC awakening in the endothelial niches, together with other factors (**Figure 3**; Risson et al., 2020).

Tumor-derived EVs also contribute to tumor angiogenesis and the disruption of the endothelial vascular barrier. Cell-to-cell communication between cancer cells and endothelial cells via EVs, is one of the most relevant mechanisms promoting vascular leakiness, angiogenesis and vascular remodeling reported in the literature (Kikuchi et al., 2019). Tumor-derived EVs, carry proteins like Tspan8 (Nazarenko et al., 2010), soluble E-cadherin (Tang et al., 2018), or Angiopoietin II (Xie et al., 2020) that are directly involved in the activation of the endothelium, or indirectly through the induction of pro-vasculogenic phenotypes in stromal cells (e.g., fibroblasts) (Cho et al., 2012; Chowdhury et al., 2015) or pro-angiogenic immune cells (Peinado et al., 2012). Breast cancer-derived exosomes expressing the cell migration-inducing and hyaluronan-binding protein (CEMIP) induce endothelial cell branching and inflammation in brain endothelial and microglial cells, upregulating the pro-inflammatory cytokines Ptgs2, Tnf, and Ccl/Cxcl that have been previously implicated in brain vascular remodeling and metastasis (Rodrigues et al., 2019). Similarly, the shedding of RNAs by EVs appears to be one of the most important mechanisms involved in angiogenesis associated with a wide variety of tumors (Skog et al., 2008; Umezu et al., 2014; Conigliaro et al., 2015; Zeng et al., 2018; Deng et al., 2020). Together, these studies suggest that tumor-derived EVs are important regulators of angiogenesis, a process that may be triggered after tumor cell awakening in endothelial cell niches (**Figure 3**).

Role of EVs on ECM Remodeling

Another hallmark of PMN formation that may be also relevant to tumor cell awakening is ECM remodeling (Peinado et al., 2017). Fibronectin deposition directly correlates with reactivation of proliferative processes in dormant cells (Aguirre-Ghiso et al., 2001). Therefore, tumor-derived exosomes may contribute to DTC awakening by altering the ECM components and provoking abnormal deposition of molecules like fibronectin

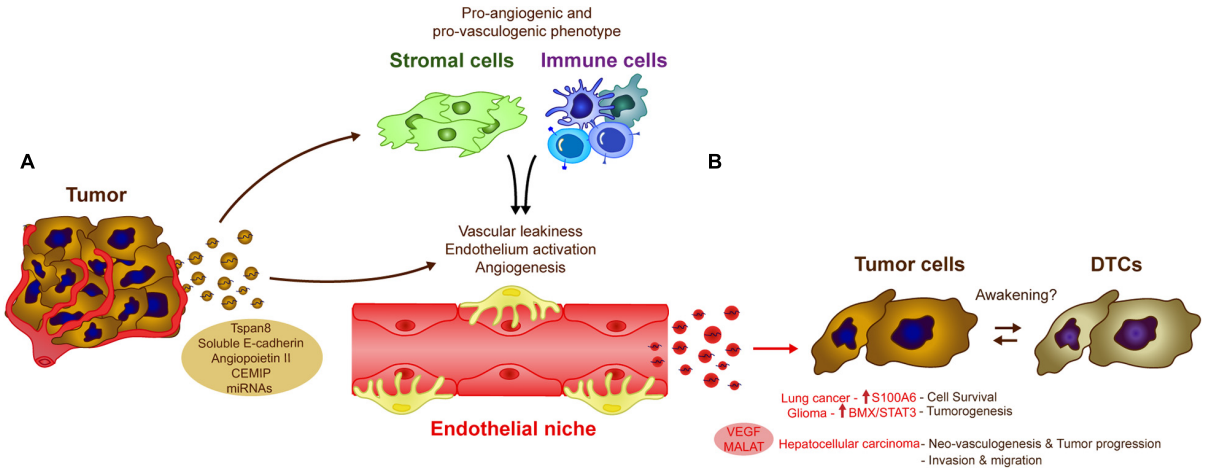

FIGURE 3 | Influence of endothelial cell-derived EVs in tumor cells. The crosstalk between tumor and endothelial cells via EVs modulate several biological processes that could influence DTC awakening. **(A)** Tumor-derived exosomes induce vascular leakiness, endothelium activation and angiogenesis, key processes in metastasis development, directly by transferring different molecules and miRNAs to the endothelial cells or indirectly by affecting other cell populations in the microenvironment as stromal or immune cells. **(B)** The altered endothelium is also capable of generating EVs, which induce pro-metastatic features (e.g., cell survival, neoangiogenesis, invasion) in a variety of cancer types (as Lung cancer, glioma, or hepatocellular carcinoma), these signals may be similar to those involved in tumor DTC awakening.

(Mu et al., 2013; Costa-Silva et al., 2015; Sung et al., 2015), collagen, laminin (Mu et al., 2013), annexins, and integrins (Hoshino et al., 2015; Keerthikumar et al., 2015). These modifications are crucial to recruit inflammatory cells, which eventually contribute to ECM degradation by secreting proteinases into metastatic niches, particularly MMPs (Rucci et al., 2011). Indeed, tumor-derived exosomes induce MMP activity, aiding ECM remodeling (Shimoda, 2019; Deep et al., 2020). Proteomic analysis has shown that these proteases may be directly transported by EVs from both the tumor and the microenvironment, playing a direct role in ECM remodeling (Taraboletti et al., 2002; Sanderson et al., 2019; Shimoda, 2019). Therefore, it is likely that these activities could be involved in the contribution of local niches to DTC awakening.

The Role of EVs in the Recruitment of Bone Marrow-Derived Cells and Local Inflammation

Another important mechanism for the generation of PMNs relies on the effect of tumor secreted exosomes on the immune system (**Figure 4**). Abnormal mobilization of immune cells, as well as the ability to switch them toward a pro-metastatic phenotype, contribute to the progression of different tumor types. The ability of tumor-derived EVs to modify homeostasis of the immune system and induce pro-inflammatory signals in different tissues facilitates both tumor and immune cell recruitment. An initial analysis of melanoma-derived exosomes demonstrated that they are involved in the mobilization of bone marrow-progenitor cells to PMNs, reinforcing metastatic behavior (Peinado et al., 2012). Melanoma-derived exosomes reprogram bone marrow progenitors toward a pro-vasculogenic phenotype, which involves the oncogene c-Met, and they induce pro-inflammatory genes in the lungs to favor the generation of PMNs (Peinado et al., 2012). Later, it was found that the generation of PMNs in the liver is promoted by Kupffer cells taking up pancreatic cancer-derived exosomes carrying MIF (Costa-Silva et al., 2015). Exosome uptake triggers the secretion of TGFβ, which in turn activates fibronectin production and deposition by hepatic stellate cells. This fibrotic microenvironment attracts bone marrow derived F4/80^{+} macrophages, which eventually enhance metastasis (Costa-Silva et al., 2015). It was later found that pro-inflammatory niches could be induced by reinforcing the expression of different S100 proteins in specific organs (Hoshino et al., 2015). Indeed, exosomes expressing integrins α6β4 and α6β1 preferentially home to the lung, while exosomes expressing integrin αvβ5 home to the liver, reinforcing PMN formation, as well as S100 protein induction and metastasis in those organs (Hoshino et al., 2015). *In vivo* treatment with exosomes derived from the EO771 model breast cancer cells also modifies the proportions of immune cells in the lungs. These EVs increase the frequency of macrophages and myeloid derived suppressor cells (MDSCs), while diminishing the numbers of CD8 T cells and NK cells, skewing the microenvironment toward an immunosuppressive state and promoting metastasis (Wen et al., 2016). In addition, recent evidence reinforces the importance of specific molecular cargos. In contrast to highly metastatic tumors, EVs derived from poorly aggressive melanomas appear to recruit completely different populations of immune cells to the PMNs, promoting immunosurveillance based on the expansion of Ly6C^{low} patrolling monocytes and NK cell recruitment, a phenomenon that results in cancer cell clearance and that thereby averts metastasis (Plebanek et al., 2017).

Exosomes exert different effects on immune cell behavior that could awaken DTCs. Besides the tumor exosomes ability to mobilize bone marrow derived cells (Peinado et al., 2012; Huan et al., 2015), they may also stimulate dormant DTCs in the bone marrow itself (Vora et al., 1994). Apart from the already mentioned mechanism of immune recruitment upon ECM remodeling (Erler et al., 2009; Yan et al., 2010;

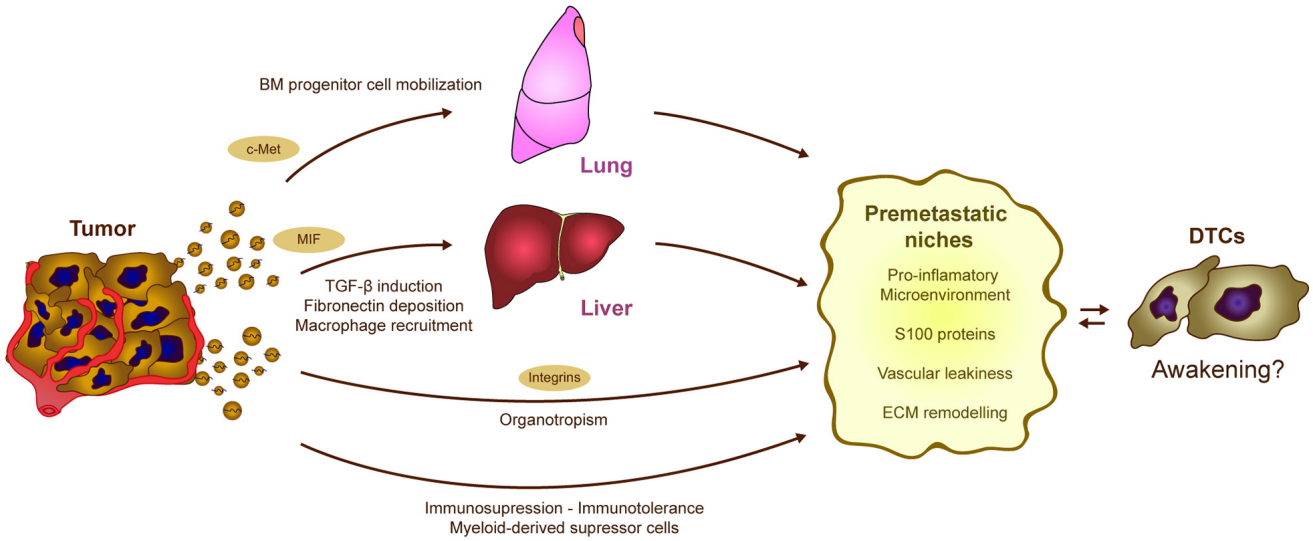

FIGURE 4 | Potential contribution of tumor-EVs to DTC awakening. The processes regulated by tumor derived EVs in formation of PMNs could be involved in DTC awakening. Tumor exosomes induce the mobilization of bone marrow precursors, which eventually contribute to the generation of pro-inflammatory and immunosuppressive niches. Similarly, alterations in the stromal cells of the liver result in the generation of a fibrotic and pro-tumorigenic microenvironment. Moreover, both PMN and sleepy niches are organotropic processes that occur at specific locations along the body depending on the tumor type. Interestingly, the expression of different integrins on the EVs surface seems to mediate metastatic organotropism. Overall, tumor derived exosomes trigger different processes as the induction of pro-inflammatory microenvironments, vascular modification, and EMC remodeling which result into the generation of optimal pre-metastatic niches. These processes may affect the equilibrium between dormancy and awakening in the DTCs.

Costa-Silva et al., 2015), exosomes can directly stimulate the stroma to actively secrete chemotactic signals, as happens in alveolar cells via Toll-like receptor 3 activation (Liu et al., 2016) or in MSCs through ERK1/2 and AKT induction (Nakata et al., 2017). Once within the PMN, tumor exosomes have the ability to modulate the immune response toward immunotolerance via different mechanisms, as extensively reviewed elsewhere (Whiteside, 2016a; Olejarz et al., 2020). To do so, tumor exosomes can block the differentiation of myeloid and lymphoid progenitors, promote pro-tumorigenic macrophage polarization, or contribute to the expansion of immunosuppressive populations like Treg lymphocytes or MDSCs (Whiteside, 2016a). The combination of immune cell recruitment and the induction of an immunosuppressive microenvironment together with ECM remodeling and vascular leakiness are key factors in PMN formation, strongly modulated by tumor EVs. All these alterations may eventually compromise immunosurveillance and disrupt tumor cell dormancy, preceding metastatic outgrowth (**Figure 4**; Muller et al., 1998; Baxevanis and Perez, 2015). However, the specific implications in DTC re-awakening still remain to be elucidated.

EXOSOMES AS BIOMARKERS OF DTC DORMANCY AND AWAKENING: THE FUTURE OF LIQUID BIOPSIES

Exosomes are considered potential biomarkers in oncological diseases (Whiteside, 2016a; Huang and Deng, 2019), and there is evidence that analyzing the exosomes in liquid biopsies could be a promising technique to detect mutations or to monitor residual disease in different biofluids (Whiteside, 2016b; Castellanos-Rizaldos et al., 2018). Indeed, a recent report showed that EVs carry specific biomarkers of cancers that can be identified with high sensitivity and efficiency in cancer patients (Hoshino et al., 2020). Hence, defining molecular signatures in liquid biopsies from patients may help identify cancer patients with a higher risk of relapse. Taking into account the non-invasive and easy ways that liquid biopsies can be obtained (Gold et al., 2015), this concept could also be applied to monitor the risk of late relapses in disease-free cancer patients. Although tumor dormancy presents challenges for clinical diagnosis and a great opportunity for therapeutic approaches, there are few studies that have focused on new methods to detect dormancy early. Defining and detecting dormant-related signatures, or pro-inflammatory and pro-awakening signatures, should be explored as clinic criteria to manage patients prior to disease reappearance (Console et al., 2019), complementing those currently existing (Sansone et al., 2017).

Liquid biopsy is defined by the analysis of biological material of tumor origin that extravasate to body fluids. Most common liquid biopsy are focused on the use of circulating tumor cells (CTCs) or circulating tumor-derived factors, in particular, circulating tumor DNA (ctDNA) (Alix-Panabières and Pantel, 2016; Husain and Velculescu, 2017; Perakis and Speicher, 2017; Pantel and Alix-Panabières, 2019). The quantification of circulating DNA molecules or CTCs showed *per se* prognostic value in many cancers (Haber and Velculescu, 2014). Another advantage of using CTCs and ctDNA resides mainly in the possibility of testing specific mutations, methylation profiles, and other DNA patterns (ctDNA and CTCs) and alternatively,

proteins and the possibility of generating patient-derived xenografts (PDX) from the most aggressive cells in the tumor that putatively could initiate metastatic outgrowth (CTCs) (Alix-Panabières and Pantel, 2016; Husain and Velculescu, 2017; Perakis and Speicher, 2017; Pantel and Alix-Panabières, 2019). Furthermore, the development of high sensitivity and specificity techniques enabled the identification of minimal residual disease (MRD) in cancer patient's follow-up blood samples (Pantel and Alix-Panabières, 2019). Complementary to these biomarkers, EVs emerged in the last years as powerful biomarkers to provide information about the tumor and the systemic changes occurring during the disease.

Due to their heterogeneous content (protein, nucleic acids, lipids, metabolites, etc.), their ubiquitous production by body cells and detection in most biological fluids, circulating EVs could be useful for specific or multiplatform analyses to provide an accurate evaluation of cancer disease at early time points, during progression, therapy and post-treatment facilitating the detection of MRD and relapse anticipation (LeBleu and Kalluri, 2020).

Each technique has its own pros and cons. For example, CTCs have been mainly used to understand the biology of early metastatic spread and resistance to established therapies, they require higher amount of plasma and specific equipment for isolation and detection (Pantel and Alix-Panabières, 2019). ctDNA analysis in plasma of cancer patients allows the identification of genomic alterations, monitoring of treatment responses, unraveling therapeutic resistance, and potentially detecting disease progression before clinical confirmation is obtained (Pantel and Alix-Panabières, 2019). In recent years a huge effort has been made to compare ctDNA and EV-DNA in order to provide a better understanding of their applications. An important limitation of EV-DNA studies is that, while ctDNA analysis is already standardized in the clinic with multiple platforms, there is a lack of standardized isolation methods of EV-DNA analysis that require clinical validation (Théry et al., 2006; Royo et al., 2020). While EV-DNA is more stable and less fragmented (Kalra et al., 2013) than ctDNA (Mouliere et al., 2011; Cheng et al., 2016; Lazaro-Ibanez et al., 2019), to date, most liquid biopsy have been performed in ctDNA (Cristiano et al., 2019; Poore et al., 2020; Sprang et al., 2020). Finally, the main advantage of EVs-based liquid biopsy is the possibility to concentrate the circulating material by specific protocols and perform multiplexing analyses of DNA with other EV cargo such as RNA that can provide a highly accurate information about the disease and will facilitate the use of personalized medicine approaches (LeBleu and Kalluri, 2020; García-Silva et al., 2021).

In the particular case of dormancy, distinguish circulating dormant cells could represent an arduous task due to the limited number of these cell populations. Similarly, defining the EV signatures related to "dormant" and "awakened" tumor cells is far from their use in the clinic. Since miRNAs in circulating EVs have diagnostic and/or prognostic potential for many cancer types, this may be a potential way to identify novel biomarkers. In exosomes isolated from plasma/serum of cancer patients, miR17-92a and miR-19a are correlated with increased colon cancer recurrence or a worse prognosis (Matsumura et al., 2015). Exosomal circulating miR141 and miR375 have been associated with metastatic prostate cancer and treatment outcome (Zedan et al., 2020), while high levels of exosomal miR-21 can predict esophageal cancer recurrence and distal metastasis (Liao et al., 2016), and this phenomenon is associated with cisplatin resistance in ovarian cancer (Pink et al., 2015). Alternatively, the down-regulation of miR-125b is correlated with metastatic melanomas (Alegre et al., 2014). Since stromal cells may fulfill a decisive role in supporting tumor dormancy through the release of miRNA containing EVs, it is tempting to speculate that analyzing the expression of miRNAs in circulating EVs could serve as a biomarker for "dormant stages" (Ono et al., 2014; Bliss et al., 2016; Vallabhaneni et al., 2017), nevertheless this is just a hypothetical scenario at this time. Since the detection of DTCs and their EV signatures is proving difficult due to their relative low abundance in circulation, defining dormant signatures based on stromal EVs could represent a more effective approach due to the concentration of material obtained after EV isolation protocols.

Similarly, defining an EV signature related to DTC awakening could potentially be of interest. While CTCs have been used successfully in the last years to detect early metastatic cell awakening, there are several limitations in sensitivity that must be solved in the case of EVs to play a relevant role in this scenario (Pantel and Alix-Panabières, 2019). Due to the strong connection between awakening and PMN formation, these combined studies could also include certain exosomal markers of PMN, some of which have already been correlated with poor prognosis, such as c-MET in the case of lung metastasis in melanoma patients (Peinado et al., 2012) or MIF in the case of liver metastasis of pancreatic cancer (Costa-Silva et al., 2015).

One of the main advantages of using liquid biopsies to detect awaked DTCs is that can be identified in pre-symptomatic patients after surgery, opening the possibility of using them as an early marker and screening tool of MRD. In addition, non-invasive liquid biopsies confer the advantage and the opportunity to follow patients during and after treatment, providing an accurate and real time read-out of tumor development with a minimal risk to patients. CTCs and ctDNA have demonstrated their use for MRD detection in plasma (Pantel and Alix-Panabières, 2019). In addition to these techniques, we recently examined the use of DNA mutations in EVs and ctDNA isolated from the drainage implanted post-lymphadenectomy to detect MRD in melanoma patients (Garcia-Silva et al., 2019). These approaches suggest that combination of several fractions (e.g., ctDNA and EV-DNA/RNA), together with the use of novel biofluids anatomically closer to tumor sites (seroma from drainage post-lymphadenectomy) and specific isolation methods (that allows the concentration of material), increases the sensitivity of detection.

Defining the signals released by the awakened DTCs could define novel biomarkers that help to identify MRD. However, liquid biopsies to determine dormancy have several limitations. First, since DTCs remain at low numbers within niches, the representation of their EVs in the bloodstream could be confused with EVs released from normal tissues. Second, it is not clear whether all tumor sub-clones at different metastatic locations will secrete and distribute EVs equally into the bloodstream, or if this

might be influenced by other factors, such as the extravasation properties of the tissues they have colonized. Third, there is heterogeneity in the dormant niches within tissues in terms of how their surrounding cell microenvironment promotes the quiescence or reawakening of DTCs. Fourth, works shown in this section come from patients that had already reactivation of the residual disease and disruption of dormancy. In order to detect signals of tumor reawakening by liquid biopsy a strong effort should be made to design new protocols and collect samples (e.g., plasma) in a routinely basis (e.g., yearly) after surgery to follow up disease reactivation.

Thus, it is important to expand the dormant EV signatures to stromal EVs rather than just limiting these to tumor-derived dormant EVs. Moreover, optimized protocols for EV isolation and cargo analysis are required, including the use of novel biofluids or combined sources [e.g., CTCs, ctDNA with EV-DNA (Garcia-Silva et al., 2019)].

CONCLUDING REMARKS

Despite the potential of exosomes to regulate a wide variety of biological and pathological processes, there is still little information regarding their involvement in tumor cell dormancy and awakening. As revised here, there are only a few studies that support this idea, the main limitation when interpreting these studies is that they are normally performed *in vitro*. Thus, it is necessary to overcome this limitation and demonstrate their relevance *in vivo*. In addition, metastasis in human patients has traditionally been addressed once the secondary lesion has been detected, meaning that dormancy had already been disrupted. Hence, human data concerning DTC awakening is very scarce (Linde et al., 2016) and the relevance of EVs remains to be defined.

Importantly, more data is needed to frame these ideas in a clinical context. Are the stromal EVs relevant in patients carrying premalignant lesions? or in patients with already invasive primary tumors? The majority of papers shown favor more the later since they mainly used evolved tumor cells (e.g., MDA-231 cells). Another main question to be solved is whether dormant niches may be influenced by naïve stroma-derived EVs or alternatively if dormant niches may be influenced by stroma-derived EVs after being primed by exosomes from primary tumors or primed by the first arriving DTCs.

Regarding the involvement of endothelial cell-derived EVs in DTC awakening, works do not clarify if these interactions are happening during early or late DTC dissemination. The clinical context is also hard to depict for these interactions. How it is possible that EVs may be relevant years after tumor resection? Is pre-metastatic niche once created able to lasts for years, even decades? Can tumor cells be influenced by endothelial cell-derived EVs in this setting? or Can DTC secrete enough EVs to trigger their own awakening? Are external factors involved (e.g., stress, neutrophil extracellular traps (NET) formation)? While both awakening and PMN formation involve tumor cells and their microenvironment, the molecular mechanisms involved in DTC awakening and the involvement of EVs have yet to be formerly demonstrated.

Another crucial issue that remains to be demonstrated is the potential to detect tumor cell awakening-derived EVs in liquid biopsies. The identification of biomarkers to detect patients with an active disease due to DTC awakening could have a major impact in the clinic. While this is plausible, several limitations must be overcome, and as such, it will be necessary to identify biomarkers secreted by "awakened" DTC that can be assessed and to confirm that they can be detected in liquid biopsies. Similarly, their correlation with disease outcome must be defined.

Thus, it is still early to determine the true potential of liquid biopsies to detect tumor cell awakening or their derived EVs. Similarly, defining the relevance of EVs derived both from tumor and stromal cells in tumor cell awakening and therapy resistance would help establish their relevance in the clinical setting.

AUTHOR CONTRIBUTIONS

HP developed the idea and wrote the manuscript. AH-B wrote the manuscript and designed the figures. LN wrote the manuscript. All authors contributed to the article and approved the submitted version.

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GLOSSARY

Abbreviation: Description
Akt: AKT; serine/threonine Kinase
AML: Acute myeloid leukemia
Ang1: Angiopoietin 1
BCR-ABL: Breakpoint cluster region/Abelson protooncogene translocation (a.k.a Philadelphia chromosome)
BM2: Breast cancer cell line BM2 (derived from the breast cancer cell line MDA-MB-231)
BMP4: Bone morphogenetic protein 4
BMP7: Bone morphogenetic protein 7
BMSC: Bone marrow stem cell
BMX: BMX non-receptor tyrosine kinase
CAFs: Cancer-associated fibroblasts
Ccl: C-C Motif Chemokine Ligand
CCR4: C-C motif chemokine ligand 4
$CD4^+$ T cells: Helper T lymphocytes
$CD8^+$ T cells: Cytotoxic T lymphocytes
CD9: Cluster of differentiation 9
CD39: Cluster of differentiation 39
CD80: Cluster of differentiation 80
CDH1: Cadherin 1
Cebp-β: CCAAT enhancer binding protein beta
CEMIP: Cell migration inducing hyaluronidase 1
CK19: Cytokeratin 19
c-Kit: KIT proto-oncogene; receptor tyrosine kinase
c-Met: MET proto-oncogene; receptor tyrosine kinase
c-Myb: MYB proto-oncogene; transcription factor
CRC: Colorectal cancer
CSCs: Cancer stem cells
CTCs: Circulating tumor cells
ctDNA: Circulating tumor DNA
CTLA-4: Cytotoxic T-lymphocyte associated protein 4
Cxcl: C-X-C motif chemokine ligand
CXCR4: C-X-C motif chemokine receptor 4
CXCL8: C-X-C motif chemokine ligand 8
CXCL9: C-X-C motif chemokine ligand 9
CXCL10: C-X-C motif chemokine ligand 10
CXCL12: C-X-C motif chemokine receptor 12
DKK1: Dickkopf WNT signaling pathway inhibitor 1
DTCs: Disseminated tumor cells
ECM: Extracellular matrix
EDG2: Lysophosphatidic acid receptor
EO771: Breast cancer cell line EO771
ER: Endoplasmic reticulum
ERK: Extracellular signal-regulated kinase
ERK1/2: Extracellular signal-regulated kinase 1/2
EVs: Extracellular vesicles
F4/80: A.k.a ADRE1 Adhesion G Protein-Coupled Receptor E1
FBXW7: F-Box and WD repeat domain containing 7
GAS6: Growth arrest-specific protein 6
G-CSF: Granulocyte colony-stimulating factor
GFAP: Glial fibrillary acidic protein
H2BK: Histone cluster 1 H2B family member K
HepN: Hepatic niche
Hoxa-9: Homeobox A9
HSCs: Hematopoietic stem cells
HUVEC: Human umbilical vein endothelial cells
IFN-γ: Interferon gamma

IL-6: Interleukin-6
IL-8: Interleukin-8
lEVs: Large extracellular vesicles
Ly6C: Lymphocyte antigen 6 complex
M1: Macrophage phenotype 1
M2: Macrophage phenotype 2
MALAT1: Metastasis associated lung adenocarcinoma transcript 1
MARKS: Myristoylated alanine-rich C kinase substrate
MCP-1: Monocyte-chemotactic protein-1
MDA-231: Breast cancer cell line MDA-231
MDA-MB-231: Breast cancer cell line MDA-MB-231
MDSCs: Myeloid derived suppressor cells
MHC-I: Major histocompatibility complex-I
MICA: MHC class I polypeptide-related sequence A
MICB: MHC class I polypeptide-related sequence B
MIF: Macrophage migration inhibitory factor
miRNAs: MicroRNA
MMP-9: Matrix metallopeptidase 9
MRD: Minimal residual disease
MSCs: Mesenchymal stem cells
mtDNA: Mitochondrial DNA
mTOR: Mechanistic target of rapamycin kinase
NK: Natural killer
Notch2: Notch Receptor 2
p38-MAPK: p38 mitogen-activated protein kinase
PDGFB: Platelet derived growth factor subunit B
PD-L1: Programmed death-ligand 1
PMN: Pre-metastatic niche
Ptgs2: Prostaglandin-endoperoxide synthase 2
PVN: Perivascular niche
S100: S100 calcium binding proteins
S100A16: S100 calcium binding protein A16
SCF-1: Stem cell factor
SDF-1: Stromal cell–derived factor 1
SDHB: Succinate dehydrogenase subunit B
sEVs: Small extracellular vesicles
STAT3: Signal transducer and activator oft transcription 3
T47D: Breast cancer cell line T47D
TGFβ: Transforming growth factor-β
THBS1: Thrombospondin-1
TIMP3: Tissue Inhibitor of metalloproteinases 3
Tnf: Tumor necrosis factor
TNFR1: Tumor necrosis factor receptor superfamily member 1A
Tregs: Regulatory T lymphocytes
Tspan8: Tetraspanin 8
UBE2N/Ubc13: Ubiquitin conjugating enzyme E2 N
ULBP1: UL16 binding protein 1
UPR: Unfolded protein response
VCAM-1: Vascular cell adhesion molecule-1
VEGF: Vascular endothelial cell growth factor
VEGF-A: Vascular endothelial growth factor A
WT: Wild-type
YAP1: Yes1 associated transcriptional regulator
ZEB2: Zinc finger E-box binding homeobox 2
α6β1: Integrin subunit alpha 6/beta 1
α6β4: Integrin subunit alpha 6/beta 4
αvβ5: Integrin subunit alpha v/beta 5.

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REVIEW
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From Good to Bad: The Opposing Effects of PTHrP on Tumor Growth, Dormancy, and Metastasis Throughout Cancer Progression

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Parathyroid hormone related protein (PTHrP) is a multifaceted protein with several biologically active domains that regulate its many roles in normal physiology and human disease. PTHrP causes humoral hypercalcemia of malignancy (HHM) through its endocrine actions and tumor-induced bone destruction through its paracrine actions. PTHrP has more recently been investigated as a regulator of tumor dormancy owing to its roles in regulating tumor cell proliferation, apoptosis, and survival through autocrine/paracrine and intracrine signaling. Tumor expression of PTHrP in late stages of cancer progression has been shown to promote distant metastasis formation, especially in bone by promoting tumor-induced osteolysis and exit from dormancy. In contrast, PTHrP may protect against further tumor progression and improve patient survival in early disease stages. This review highlights current knowledge from preclinical and clinical studies examining the role of PTHrP in promoting tumor progression as well as skeletal and soft tissue metastasis, especially with regards to the protein as a regulator of tumor dormancy. The discussion will also provide perspectives on PTHrP as a prognostic factor and therapeutic target to inhibit tumor progression, prevent tumor recurrence, and improve patient survival.

Keywords: PTHrP, bone metastasis, dormancy, hypoxia, proliferation

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INTRODUCTION

The initial discovery of parathyroid hormone- related protein (PTHrP) came about from studies on humoral hypercalcemia of malignancy (HHM), a paraneoplastic syndrome in which elevated levels of PTHrP lead to increased osteoclastic bone resorption and serum calcium levels (1, 2). HHM is most commonly diagnosed in patients with advanced-stage lung, renal, and neuroendocrine tumors. Though less frequently, breast cancers can also cause HHM. While hypercalcemia had been associated with cancer since the early twentieth century (3), it was Fuller Albright in 1941 who first postulated that this complication may be caused by tumor secretion of parathyroid hormone (PTH) or another similar factor due to its known roles in calcium homeostasis (4). While functionally this hypothesis seemed likely, clinically this did not prove to be true based on differences in the

clinical presentation of patients with hypercalcemia due to cancer vs. those with hypercalcemia due to known PTH excess syndromes like primary hyperparathyroidism (5). By the 1980s multiple independent groups had eventually purified a protein similar in structure and biological function to PTH from human lung cancer (6), breast cancer (7), and renal cell carcinoma cell lines (8). This 18 kDA protein, now termed PTHrP, was found to have very high homology with the amino-terminal region of PTH such that eight of the first 13 residues are identical (9).

The role of PTHrP in cancer now extends well beyond its role in HHM. PTHrP is a well-established critical mediator of tumor-induced osteolysis, especially in breast cancer, which has a high tropism for disseminating to the bone marrow. In fact, ∼70% of patients who succumb to breast cancer display evidence of bone metastases on postmortem examination (10). Lung cancer, melanoma, renal cell carcinoma, and thyroid cancers also metastasize to bone with relatively high (>20%) frequency (11) and form osteolytic lesions (12). Bone-disseminated tumor cells secrete PTHrP (13–15), which drives bone destruction *via* stimulation of receptor activator of NFκB ligand (RANKL)-mediated differentiation and activation of osteoclasts (16). Osteoclasts resorb the bone matrix, releasing numerous pro-tumorigenic factors such as transforming growth factor beta (TGFβ), matrix metalloproteinases, and other growth factors that subsequently fuel tumor cell proliferation and more PTHrP secretion (17, 18). PTHrP expression by bone-disseminated tumor cells is also uniquely fueled by the microenvironment. The rigidity of the bone matrix activates TGF-β dependent mechanical signals that stimulate expression of both PTHrP and Gli2, a transcription factor in the hedgehog signaling pathway that in turn induces more PTHrP expression (19). The bone microenvironment provides yet another critical level of regulation of Gli2 and PTHrP expression *via* the Wnt pathway (20). Matrix rigidity activates Wnt signaling and induces nuclear β-catenin accumulation, while bone marrow stromal cells secrete canonical (including Wnt3a) and non-canonical Wnt ligands. Both processes further drive Gli2 and PTHrP transcription and eventual bone destruction.

Patients who develop bone metastases may experience severe pain, impaired mobility, pathologic fractures, spinal cord compression and hypercalcemia (21). While bisphosphonates and denosumab, inhibitors of osteoclast activity, are commonly utilized to manage metastasis-related symptoms and have been shown to decrease the incidence of bone metastases, they only improve survival in postmenopausal breast cancer patients (22, 23). No survival benefits have been observed in patients who were premenopausal at the time of diagnosis (24–26). Thus, there remains an urgent need to identify therapies that effectively target bone metastatic tumors and improve survival.

In addition to its critical role in tumor-induced bone disease, PTHrP has more recently been investigated as a potential regulator of tumor dormancy owing to its roles in regulating tumor cell proliferation, apoptosis, and survival (27) and genes that have been specifically implicated in tumor dormancy (28). Generally, dormancy can be characterized as (i) cellular dormancy where tumor cells persist as either solitary cells that are non-proliferative (e.g., Ki67 or BrdU negative) and arrested in the G0 cell cycle phase, or (ii) tumor mass dormancy where the growth capacity of micrometastases is limited due to balanced proliferation and apoptosis, suppression of angiogenesis, or immune clearance (29–31). Tumor dormancy is believed to be the cause of late recurrence even months to years following successful removal of the primary tumor (32–34). Long latency periods are mostly frequently observed in estrogen-receptor positive (ER^+) breast cancer (35, 36) as well as prostate cancer (37). Patients with other cancer types including non-small cell lung cancer (38), renal cell carcinoma (39) and colorectal cancer (40) may also exhibit late tumor recurrence, although this occurs less frequently than in breast cancer patients. Dramatic increases in breast cancer patient survival in recent decades are, in part, due to significant improvements in treating and managing primary breast tumors. Thus, it is possible that as therapies continue to improve for other cancer types that extend patient survival, longer latency periods may also be observed. Tumor dormancy presents a significant clinical dilemma as these dormant DTCs not only have the potential to become reactivated and proliferate into a macrometastasis, but also evade immune surveillance and anticancer therapy (41, 42). Currently there are no cures for metastatic disease or therapies to prevent tumor exit from dormancy and late recurrence. This review will explore current knowledge from preclinical and clinical studies regarding the role of PTHrP in promoting skeletal and soft tissue metastasis. We will also explore findings regarding PTHrP as a regulator of tumor dormancy and provide our perspective on PTHrP as a therapeutic target to inhibit tumorigenesis, prevent tumor recurrence, and improve patient survival.

PTHrP BIOLOGY

PTHLH, the gene encoding PTHrP, is located on chromosome 12, and has nine exons spanning ∼15 kb with at least three identified promoters. Alternative splicing gives rise to three isoforms containing 139, 141, and 173 amino acids (43). PTHrP also has multiple domains, each with different biological functions (44). The first 36 amino acids (−36 to −1) encode a domain that controls intracellular trafficking of PTHrP precursors before being cleaved when the mature molecule is secreted. The next domain (amino acids 1–34) is responsible for PTHrP binding to and activation of the PTH receptor type 1 (PTH1R), a G-protein coupled receptor. In fact, eight of the first 13 residues within this region of PTHrP are identical with PTH, allowing the two polypeptides to exert agonist effects on their shared receptor (44, 45). The nuclear localization sequence (NLS) from amino acids 67–94 is important for intracrine actions of PTHrP in the nucleus and cytoplasm including regulating cell proliferation, survival, and apoptosis (46). Lastly, the carboxy-terminal domain beginning at residue 107 is associated with a number of identified biological actions including inhibition of osteoclast-mediated bone resorption and anabolic effects in bone *via* a region termed "osteostatin" as well as a nuclear export sequence (NES) (43, 47).

Endocrine, Autocrine, and Paracrine Activity of PTHrP

In normal physiology, PTHrP acts as a hormone to control calcium transport across the placenta to the fetus (48) and during lactation when it enters systemic circulation (49). In HHM, PTHrP secreted by tumors in the breast and lung, for instance, also acts as a hormone distantly to increase bone resorption (1, 2). The autocrine and paracrine roles of PTHrP in normal postnatal physiology have been reviewed extensively elsewhere (44, 46). Thus, several of these physiologic functions that are less pertinent to this review will only briefly be mentioned here. PTHrP is highly expressed in human tissues and plays important roles in mammary gland development, tooth eruption, keratinocyte differentiation for hair follicle development, chondrocyte maturation, and endochondral bone formation (44, 46). Perhaps one of the most well-studied paracrine functions of PTHrP is the regulation of normal bone remodeling where it is produced locally by early osteoblast progenitors to promote differentiation of mature osteoblasts and bone formation (50, 51). PTHrP also inhibits apoptosis of early and mature osteoblasts and osteocytes. Furthermore, osteoblast-derived PTHrP stimulates osteoclast differentiation to increase bone resorption. These actions of PTHrP must occur in a balanced manner to maintain the integrity of the bone. While physiologic, these paracrine functions of PTHrP can also pathologically fuel osteolysis and the growth of bone disseminated tumors as discussed previously (17, 18). Lastly, PTHrP plays a well-recognized role as a paracrine regulator of smooth muscle relaxation, particularly in the vasculature (52) where incubation with PTHrP (1–34aa) also activates cAMP production, indicating that this effect is indeed mediated through PTH1R (53, 54). In vascular smooth muscle cells, treatment with exogenous PTHrP acting through PTH1R inhibits cell proliferation (55, 56).

In addition to binding and activating PTH1R to exert its paracrine/autocrine functions, PTHrP can translocate into the nucleus when its NLS forms a complex with importin β, a nuclear transport factor, and the GTP-binding protein Ran (57). Interestingly, in vascular smooth muscle cells, intracrine actions of PTHrP localized to the nucleus paradoxically increase proliferation (56). Indeed, in A10 smooth muscle cells overexpressing wild-type PTHP, the protein localizes in the nucleus of cells that are dividing or completing cell division. This is in striking contrast to findings that PTHrP inhibits proliferation and cell cycle progression in the same cells when acting through PTH1R (55, 56). These effects of PTHrP are particularly important in the discussion of its role as a regulator of tumor dormancy as it has also been demonstrated that PTHrP lacking the NLS arrests cell cycle progression by increasing $p27^{Kip}$, a cyclin dependent kinase inhibitor, and decreasing phosphorylation of Rb (58, 59). Cell cycle arrest in the G0-G1 phase is a key characteristic of quiescent cells (60, 61) and p27 is elevated in G0 arrested cells (62, 63). These findings in vascular smooth muscle cells are remarkable as they indicate that PTHrP can have paradoxical roles on mitogenesis depending on the mode of signaling: paracrine/autocrine vs. intracrine.

In addition to nuclear localization mediated by importin β, PTHrP can also gain entry into the nucleus by other mechanisms. PTHrP can be secreted but then internalized in an autocrine/paracrine manner *via* the PTH1R before being shuttled to the nucleus (64). Secreted PTHrP may also enter the nucleus through endocytosis-dependent translocation initiated by binding with a non-PTH1R cell surface receptor (65). Another potential mechanism regulating its subcellular localization is if translation is initiated at a codon different from the classic AUG site. As a known example, translation of fibroblast growth factor-3 (FGF3) can be initiated at an AUG codon resulting in direction of the peptide for secretion (66). If translation begins at an alternative upstream CUG site, FGF3 is directed into the nucleus. Like FGF3, the PTHrP prepro region has an alternative translational start site at a CUG codon (67), which may serve a similar purpose in regulating PTHrP secretion vs. nuclear import. Since the differential localization of PTHrP produces divergent mitogenic cellular effects in vascular smooth muscle cells, the same is likely true in cancer cells, complicating the understanding of PTHrP as a regulator of cell proliferation and tumor dormancy. Consequently, if altering PTHrP nuclear localization is to be leveraged for therapeutic purposes, more investigation is needed to better understand the regulation of PTHrP subcellular localization in cancer cells and how this may change throughout tumorigenesis.

ROLES OF PTHrP IN TUMORIGENESIS, METASTASIS, AND TUMOR DORMANCY

Preclinical Evidence for PTHrP Regulation of Tumor Growth and Proliferation

Our understanding of the paracrine/autocrine and intracrine actions of PTHrP extends far beyond the physiologic activities described in the bone, vasculature, and various other normal epithelial tissues. PTHrP also modulates growth, progression, and metastasis in various cancer types by regulating: (i) cell survival, (ii) cell proliferation, (iii) apoptosis, and (iv) invasion and migration (68, 69). For example, human MCF7 breast cancer cells overexpressing PTHrP (−36 to −139) display significantly greater survival as they are protected from serum starvation-induced apoptosis and express elevated levels of the antiapoptotic proteins Bcl-2 and Bcl-xL (69). Other studies have demonstrated that PTHrP drives breast tumor growth by promoting proliferation, as demonstrated by increased staining for the proliferative markers Ki67 and cyclin D1 (61). Human breast cancer cells expressing PTHrP (−36 to −139) are also enriched in the G2/M cell cycle phase compared with cells overexpressing NLS-mutated PTHrP, indicating an intracrine role for PTHrP in regulating cell cycle progression and cell growth. In prostate cancer cells, PTHrP expression stimulates proliferation and induces intracrine production of Il-8, a known growth-promoting factor (70). Prostate cancer cells overexpressing full-length PTHrP also show significantly increased cell survival when exposed to various apoptotic agents (71). Another study determined that treatment with PTHrP

neutralizing antibodies dramatically inhibits clear cell renal cell carcinoma cell proliferation *in vitro* and induces regression of implanted tumors by inducing apoptosis *in vivo,* further indicating a role for PTHrP in regulating both proliferation and cell death (72). PTHrP also positively regulates LoVo human colon cancer cell proliferation, migration and invasion *in vitro* (73). Lastly, human cancer cells that overexpress full-length PTHrP display upregulated expression of the α1, α5, α6, and β4 integrin subunits (74), which are known to facilitate cancer cell adhesion, migration and invasion (75, 76).

PTHrP expression in the primary tumor has also been identified as an important regulator of tumor growth in *in vivo* genetic models. In the PyMT-MMTV (mouse mammary tumor virus-polyoma middle T antigen) model of breast carcinoma where mice spontaneously develop mammary tumors, Cre-loxP-mediated *Pthlh* ablation delays primary tumor initiation and inhibits tumor progression (68). Palpable tumors appear much later and measure smaller than those in control mice. Mechanistically, the authors found reductions in the expression of Ki67, factor VIII (an angiogenesis marker), Bcl-2 (an antiapoptotic protein), cyclin D1 (a cell-cycle regulator), and AKT1 (a pro-survival factor). These data indicate that in this model of breast cancer, PTHrP acts as a pro-tumorigenic factor that drives tumor cell growth and proliferation in the primary site. In striking contrast, another *in vivo* study found that Cre-mediated loss of PTHrP in the MMTV-*neu* mouse model increases tumor incidence and reduces survival (77). In comparing these discrepant results from the studies on the PyMT-MMTV mice (68) vs. the MMTV-*neu* mice (77), it is important to note that the *neu*-based model reflects late-onset oncogenesis representing tumors arising in older animals while the PyMT-MMTV-based model reflects earlier onset tumorigenesis. Age can significantly affect tumor behavior (78, 79). Thus, in these pre-clinical tumor models, age at which cancer develops must be carefully factored into the interpretation of the effects of PTHrP on tumorigenesis. Lastly, authors of the PyMT-MMTV study report that they deleted exon 4, which encodes amino acids 1–137 in mice (44). While the authors of the MMTV-*neu* study do not explicitly state which portion of the gene was targeted, deletion of a different exon or smaller portion of the gene could explain these opposing observations since targeting different domains of the PTHrP molecule can elicit distinct cellular responses.

The previously discussed *in vivo* studies all rely on mouse tumor models. However, it is important to also recognize the utility of studies investigating PTHrP using models of spontaneous cancers that develop in larger animals such as dogs and cats (80). These animal models also contribute to our understanding of the biology of PTHrP and its role in tumorigenesis in ways that are distinct from studying rodent models alone. For instance, in feline oral squamous cell carcinoma (OSCC), elevated expression of PTHrP [which displays a high degree of sequence similarity to human isoforms (81)] is associated with increased bone invasion and osteoclastogenesis (82). Interestingly, tumor cells derived from bone specimens with evidence of osteolysis have more nuclear localization of PTHrP compared to OSCC without osteolysis. This model provides a unique finding where in addition to the paracrine actions of PTHrP in the bone, the intracrine functions of the polypeptide also appear to strongly influence the osteolytic phenotype of tumor cells. A feline oropharyngeal squamous cell carcinoma cell lines (SCCF1) has also been developed that expresses elevated PTHrP mRNA and protein in response to TGF-β stimulation (83) similar to human cancer cell lines (19, 84). Elevated PTHrP expression has also been noted in numerous neoplastic canine tissues compared with normal matched tissue (85, 86). There is great potential to learn even more about the biology of PTHrP and its role in tumorigenesis using these large animal models.

PTHrP's Role in Regulating Tumor Cell Dormancy

Most pre-clinical data support a pro-tumorigenic role for PTHrP. PTHrP is also likely a negative regulator of tumor cell dormancy due to its actions that modulate proliferation, apoptosis and cell survival. One study that provides some of the most direct and striking evidence to support this found that in ER^+ human MCF7 breast cancer cells, which lie dormant *in vivo* following intracardiac injection (28, 87–89), overexpression of PTHrP (1–141) pushes these cells out of quiescence, switches them to a highly osteolytic phenotype and dramatically increases tumor burden in the bone (87). Consistent with this enhanced bone colonization and exit from dormancy, a later study determined that PTHrP (1–139) overexpression in MCF7 cells also represses expression and downstream signaling of leukemia inhibitory factor receptor (*LIFR*), a known breast tumor suppressor and dormancy factor in the bone (28). In this study, overexpression of PTHrP and loss of LIFR both enable otherwise dormant breast cancer cells to downregulate several quiescence- associated genes including thrombospondin-1 (TSP1) (90), transforming growth factor-β2 (TGF-β2) (91), tropomyosin-1 (TPM1) (92), and Selenbp1 (93), among others. Common regulation of this group of genes suggests that PTHrP may inhibit pro-dormancy signaling mediated by LIFR. Moreover, intracardiac injection of MCF7 LIFR knockdown cells into mice results in greater bone destruction *via* increased osteoclastogenesis and tumor cell proliferation (28). Thus, repression of LIFR either directly or perhaps through PTHrP overexpression can push bone-disseminated breast tumor cells out of dormancy. These data are further supported by the PyMT-MMTV genetic studies by Li et al. (68), which demonstrated that *Pthlh* ablation reduces primary breast tumor growth with reductions in pro-proliferative factors Ki67 and cyclin D1 as well as the anti-apoptotic protein Bcl-2, all factors known to regulate dormancy.

Interestingly, evidence exists suggesting that multiple breast cancer cell lines express PTH1R at varying levels, but do not activate downstream cAMP signaling in response to PTH or PTHrP, despite functional signaling in response to calcitonin and PGE_2 which serve as positive controls (94). In this study, there was also no activation of a cAMP response element reporter construct, and RNA sequencing confirmed that only 2 out of 36 genes in a previously described

panel of cAMP responsive element binding protein (CREB)-responsive genes (95) were significantly upregulated in MCF7 PTHrP-overexpressing cells. Taken together, these data provide convincing evidence that in the bone colonization models, the effects of PTHrP overexpression on gene expression, including dormancy-associated factors in MCF7 cells, are independent of PTH1R activation of the cAMP/PKA/CREB pathway. Further studies are warranted to explore non-PTH1R mediated actions, which may reveal novel mechanisms by which PTHrP negatively regulates dormancy in bone-disseminated breast tumor cells.

Lastly, other studies in breast cancer have also revealed that PTHrP may alter adhesion to extracellular matrix (ECM) cell surface receptors, which can trigger intracellular signaling that promotes cell cycle progression and exit from a dormant state (96–98). Specifically, PTHrP regulates the expression of integrins which mediate interactions between tumor cells and the ECM that can modulate cellular quiescence (99). For example, downregulation of the urokinase plasminogen activator receptor (uPAR), a known mediator of tumor dormancy *in vivo*, decreases complex formation with α5β1 integrin and cell adhesion to fibronectin (100). This reduced ECM binding consequently maintains tumor cells in a dormant state by inhibiting activation of extracellular regulated kinase (ERK) signaling, which normally functions to promote cell cycle progression and division (101). Additional studies have also confirmed that inhibiting ERK signaling *via* altered uPAR-mediated α5β1 integrin interactions promotes quiescence *in vivo* (102). This is highly relevant in the evaluation of PTHrP as a regulator of dormancy since overexpression of PTHrP (−36 to −139) in MDA-MB-231 human breast cancer increases adhesion to fibronectin (103). PTHrP (−36 to −139) overexpression in tumor cells also significantly increases mRNA and cell surface expression of various integrins including α_5, α_6, β_1, and β_4. Though it has not been directly studied, PTHrP may push tumor cells out of dormancy by inducing integrin expression, cell adhesion to fibronectin, and activation of ERK signaling. Additional studies are needed to understand how PTHrP alters ECM binding to regulate tumor dormancy.

Prostate tumors, like breast tumors, also exhibit long latency periods before micrometastases become clinically detectable (37, 92). One study found direct evidence that PTHrP promotes prostate cancer progression in the bone (104). Overexpression of PTHrP (1–87) and PTHrP (1–173) in the non-invasive DU-145 human prostate cancer cell line converted these cells to an aggressive phenotype resulting in significantly greater bone tumor burden and mixed osteolytic/osteoblastic lesions following intrafemoral injection. Interestingly, mice injected with PTHrP (1–173) cells had more extensive bone lesions than those injected with PTHrP (1–87) mice despite lower serum PTHrP levels. Not only does this study demonstrate that PTHrP expression can push prostate tumor cells out of dormancy but it also highlights the pleiotropic actions of the protein's different domains, as PTHrP (1–87) lacks the full nuclear localization sequence, osteostatin region, and mitogen regulatory sequences contained in the carboxy terminus of the full-length molecule. The effects of the carboxy terminus of PTHrP, in particular, need to be examined more extensively to specifically understand how this region promotes cancer progression in bone and regulates tumor dormancy. Another study of early prostate adenocarcinoma also demonstrated that PTHrP overexpression significantly increases primary tumor growth (105). This study found no difference in growth rates between human prostate cancer cells transfected with full-length PTHrP and vector controls, but PTHrP overexpression did render the cells less susceptible to phorbol-12-myristate-13-acetate (PMA)- induced apoptosis. Other studies have also identified a role for PTHrP in inhibiting apoptosis (106, 107). Thus, PTHrP may negatively regulate tumor dormancy by not only increasing cell proliferation, but also by disrupting the balance with cell death.

Interestingly, other *in vitro* studies, particularly on tumor cells in soft tissues have provided contrasting findings on the role that PTHrP plays in tumor dormancy. Administration of neutralizing antibodies against PTHrP (1–34) to mice inoculated with PTHrP-expressing orthotopic lung carcinomas significantly increases tumor growth (108). In a later study by the same authors on human lung adenocarcinoma lines that are normally PTHrP-negative, ectopic expression of PTHrP (1–87) induces arrest in or slows progression through G1 compared with control cells (109). Expression of cyclin D2 and cyclin A2 were also lower while expression of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor, was increased indicating that PTHrP inhibits the proliferation of lung tumor cells and may actually promote dormancy in this tumor model. It is interesting to note that in this study, as in the breast cancer study by Johnson et al. (94) discussed previously, there was no observed increase in cAMP production, making autocrine/paracrine signaling *via* PTH1R unlikely. In addition, the plasmid for PTHrP (1–87) encodes a truncated protein lacking the full NLS suggesting that extra-nuclear forms of the protein may interact with other cytoplasmic factors to regulate tumor cell proliferation. However, it is worth noting that peptides <50–60 kDA such as PTHrP (1–87) can still passively enter the nucleus without an NLS (110). Thus even truncated forms of PTHrP that lack the NLS may still localize to the nucleus. This further highlights the necessity of more studies to establish whether the mitogenic and dormancy effects of PTHrP depend on autocrine/paracrine, or intracrine mechanisms.

The studies on breast, prostate and lung cancer discussed in the previous sections do present mixed findings regarding the role of PTHrP in regulating tumor growth and dormancy. This would suggest that the actions of PTHrP are highly dependent on the tumor type and microenvironment. In the bone, tumor cell autonomous actions of PTHrP promote emergence from a quiescent state (28, 87, 104). This may be complemented by paracrine actions of tumor-secreted PTHrP on bone marrow stromal cells like osteoclasts that promote the release of additional pro-tumorigenic factors to further increase tumor growth. However, in tumors and metastases that arise in other soft tissues, the opposite may be true. This is also evident in another *in vivo* small cell lung cancer study where administration of an anti-PTHrP antibody significantly inhibits bone metastasis formation, but not metastasis to visceral organs (lungs, liver, kidneys, lymph nodes) (14). This suggests that PTHrP may uniquely drive metastasis formation in the bone, but

not other soft tissues. Clinical evidence of PTHrP's unique role in metastasis to bone vs. soft tissues will be discussed further in later sections. This is particularly important as the potential success of PTHrP targeted therapies will depend on careful selection of patients with tumor types at highest risk for recurrence in organs where its expression actually drives exit from dormancy and metastatic outgrowth.

PTHrP's Role in Regulating Tumor Mass Dormancy

In addition to modulating cellular dormancy, PTHrP's role in regulating angiogenesis and immunosurveillance, the two key mechanisms that characterize tumor mass dormancy, must also be considered. Angiogenesis is critical as tumors generally cannot exceed 2–3 mm in diameter without developing new blood vessels or co-opting pre-existing vasculature to avoid growth-limiting oxygen deprivation due to hypoxia (low oxygen tensions) and nutrient deprivation (111). Importantly, the bone marrow is a physiologically hypoxic microenvironment (112, 113) and hypoxia is evident in most solid tumors (114). Angiogenic dormancy results when insufficient vascularization induces cell death that counterbalances the rate of proliferation, resulting in no net growth of the tumor mass (60, 115). Emergence from dormancy and tumor progression may resume after an "angiogenic switch" in which there is a shift in the balance between pro-angiogenic factors [e.g., vascular endothelial growth factor (VEGF)] and anti-angiogenic factors (e.g., thrombospondin-1) (116). Consequently, pro-angiogenic signaling dominates and new blood vessels form.

Several studies have investigated the effects of PTHrP on tumor-induced angiogenesis, though the results are conflicting. Early work by Bakre et al. demonstrated that PTHrP inhibits endothelial cell migration *in vitro* and angiogenesis in prostate tumors *in vivo* through activation of protein kinase A (117). Consistent with this inhibitory effect, PTHrP reduces VEGF production during osteoblast differentiation and endochondral bone formation (118). These results suggest that PTHrP may prevent tumor growth by inducing angiogenic dormancy. However, numerous other studies have demonstrated that PTHrP stimulates tumor-induced angiogenesis. PTHrP increases expression of pro-angiogenic factors including VEGF (119), and factor VIII (68) in breast cancer bone metastases. In prostate cancer cells PTHrP overexpression stimulates IL-8 production, another key pro-angiogenic factor (70). Malignant pituitary tumor cells that overexpress PTHrP also induce neovascularization in xenografts *in vivo* (120). Mechanistically, recombinant PTHrP (1–34) increases capillary formation by endothelial cells through PTH1R activation and cAMP signaling. Another study found that exogenous PTHrP treatment *in vivo* increases expression of VEGF and CD31 (a marker of vascular endothelial cells) in colorectal tumors (118).

Overall, these studies indicate that PTHrP promotes tumor-induced angiogenesis, making it plausible that the protein could act as a key negative regulator of tumor dormancy by stimulating new vessel formation. Conflicting findings are likely due to diversity within the tumor microenvironment where there are different target cells of PTHrP that each may individually regulate angiogenesis. Moreover, different domains and biologically active fragments of PTHrP likely will have differing effects on endothelial cells and other stromal cells during angiogenesis, but these studies did not explore differences between the different PTHrP isoforms. Lastly, it is important to note that while angiogenesis and angiogenic dormancy can be regulated by both hypoxia and PTHrP activity, PTHrP is also regulated by hypoxic signaling. Studies in chondrocytes determined that PTHrP expression is induced by hypoxia in a HIF1α (hypoxia inducible factor 1 alpha) and HIF2α (hypoxia inducible factor 2 alpha) dependent manner (121). However, it has been show in prostate cancer cells that while HIF1α and HIF2α are both able to bind to the *PTHLH* promoter, only HIF2α induces transcription (122). As hypoxia has been shown to have dual roles in both promoting and negatively regulating quiescence (123, 124), PTHrP's complex role in angiogenesis may be yet another mechanism by which low oxygen tensions differentially regulate tumor dormancy.

Immunosurveillance plays a well-characterized role in suppressing tumor growth and maintaining micrometastases in a dormant state (125). Components of the adaptive immune system including $CD4^+$ (126, 127) and cytotoxic $CD8^+$ (128) T cells are key players known to limit the outgrowth of dormant disseminated tumor cells (129). Natural killer (NK) cells are a pivotal component of the innate immune system that can maintain tumors in a dormant state by both their cytotoxic activity as well as stimulation of anti-tumorigenic cytokine production by $CD4^+$ and $CD8^+$ T cells (130, 131). In contrast, regulatory T cells (Tregs) are associated with immune suppression and tumor progression in numerous cancer types (132, 133). Lastly, the myeloid-derived suppressor cells (MDSCs) are a unique subpopulation of immature myeloid cells that play a prominent role in reactivating dormant disseminated tumor cells and promoting metastatic outgrowth by promoting immune suppression and angiogenesis (134, 135). While few studies have examined the role of PTHrP in modulating tumor infiltration of each of these immune cell types, a few have specifically examined the MDSCs that are identified by the expression of myeloid cell (CD11b) and granulocytic (Gr-1) markers (136). One study found that treatment with recombinant PTHrP or overexpression of the protein both promote the recruitment of $CD11b^+Gr1^+$ MDSCs into prostate tumor tissue where they increase primary tumor growth *in vivo* (137). In the bone marrow, tumor-derived PTHrP also promotes recruitment and activation of $CD11b^+Gr1^+$ MDSCs, resulting in increased MDSC-derived MMP-9 expression, which drives prostate cancer invasion and angiogenesis. Similar findings were demonstrated in a separate study of murine mammary carcinoma where intratumoral $CD11b^+Gr1^+$ cell recruitment enhanced metastatic outgrowth *via* increased metalloproteinase activity (138). $CD11b^+Gr1^+$ MDSCs derived from the bone marrow of breast tumor-bearing mice also have elevated expression of transforming growth factor β (TGFβ), a well-known potent stimulator of PTHrP expression, thus perpetuating the cycle of tumor-induced osteolysis that fuels tumor growth (139). Taken together, these results suggest

that PTHrP may play a critical role in negatively regulating tumor mass dormancy by increasing infiltration of immune suppressive MDSCs, which promote tumorigenesis (140, 141). PTHrP actions on recruitment of other immune populations in the tumor microenvironment have been inadequately explored. These studies are critical to gaining a more complete understanding of the role of PTHrP as a regulator of tumor mass dormancy.

Clinical Evidence for PTHrP Effects on Tumor Growth and Metastasis

Much like the *in vitro* and *in vivo* analyses, clinical studies investigating PTHrP as a prognostic factor have produced opposing findings, complicating the understanding of the role of the molecule in tumorigenesis, metastasis, and tumor dormancy. Henderson et al. conducted a large and comprehensive prospective study over 10 years in patients with breast cancer and found that positive immunohistochemical staining for PTHrP in 79% of the primary tumors was associated with significantly improved survival and decreased bone metastasis (142). These results would suggest that PTHrP decreases the invasive capacity of breast tumor cells and is protective against tumor growth in the primary site and formation of distant metastases. Interestingly, this study also revealed that of the 19 patients with bone metastases requiring surgical intervention, 7 patients had PTHrP-negative primary tumors. However, the majority of the individuals with PTHrP-negative primary tumors still developed PTHrP-positive bone lesions. All patients in the study with PTHrP-positive primary cancers also had positive expression in their bone metastases. Thus, there is not a clear inverse relationship between PTHrP expression at the primary and bone secondary sites. It is important to note this frequency of bone metastases in patients with PTHrP-negative primary breast cancers is still consistent with known tumorigenic roles for PTHrP when tumor cells colonize the bone later in disease progression. The bone marrow microenvironment enhances tumor cell production of PTHrP, which drives osteolysis and further metastatic growth (17, 18). Thus, protective PTHrP actions early in tumorigenesis at the primary site are likely distinct from its deleterious effects once disseminated tumor cells reach the bone.

Another breast cancer study that aligns with the overall conclusions of Henderson et al. (142) found that PTHrP levels are downregulated in malignant compared with normal breast epithelia, but also low levels of nuclear localized PTHrP correlate with unfavorable clinical outcomes (143). Mechanistically, the authors found a strong positive correlation between nuclear PTHrP levels and nuclear pStat5. This may explain, in part, why nuclear PTHrP is associated with the unfavorable clinical outcomes since loss of Stat5 expression and activation in breast cancer has consistently been associated with poor prognosis (144, 145). Again, this observed progressive loss of nuclear PTHrP from well-differentiated mammary epithelia to poorly differentiated, aggressive cancer cells would suggest important context-dependent roles for PTHrP signaling in tumorigenesis. In early stages, intracrine signaling of nuclear PTHrP may be protective against malignant transformation, but in distant sites like the bone, reactivation of PTHrP can still induce extensive osteolysis that would drive metastatic tumor growth.

Other clinical studies have identified protective roles of PTHrP in other solid tumor types. An analysis of non-small cell lung cancer (NSCLC) determined that PTHrP (109–141) expression in the primary tumor was associated with longer disease-free survival in female patients with either early or advanced stages of disease (146). Interestingly, female patients in this study with PTHrP-negative cancer had a shorter lifespan than all other participants, including male patients with PTHrP-negative or positive carcinomas. Thus, absence of tumor PTHrP appears to be a negative prognostic indicator specifically in women with NSCLC. The exact etiology of the sex dependence of PTHrP as a prognostic factor in lung cancer has not been further studied. However, 17β-estradiol (E_2) and tamoxifen have both been shown to regulate PTHrP expression in breast cancer cells (147), suggesting an association between estrogen receptor signaling and PTHrP during tumorigenesis. Lastly, in clear cell renal cell carcinoma (RCC), it has also been determined that decreased intensity of the carboxy-terminal region of PTHrP (amino acids 109–141) is associated with significantly greater cases of tumor recurrence (148). This would suggest a role for PTHrP in increasing recurrence free survival in patients with RCC.

By contrast, numerous other clinical studies, especially in breast cancer, have concluded that PTHrP supports tumor growth and progression. In a large analysis including two genome-wide association studies from 41 case–control studies through the Breast Cancer Association Consortium (BCAC) and nine breast cancer genome-wide association studies, *PTHLH* was identified as a susceptibility locus in both ER^+ and ER^- breast cancer (149). This study of patients with invasive breast cancer and ductal carcinoma *in situ* (DCIS) provides additional evidence implicating PTHrP in breast cancer pathogenesis, independent of its roles in promoting osteolysis. It is important to note that this analysis was performed on data from retrospective case-control studies enrolling multiple smaller patient cohorts. This factor should be kept in mind when comparing these findings with those of the better-powered, prospective study conducted by Henderson et al. (142) that identified PTHrP as a protective factor. In another study on patients with ER^+ and ER^- breast cancer, expression of both PTHrP and its receptor correlated with reduced disease-free survival while receptor expression alone correlated with reduced overall survival (150). In this study, PTHrP expression was detected by an antibody to the amino terminal region (1–34) in 68% of primary tumor specimens compared with 100% of bone metastases and the PTHrP receptor was present in 37% of tumors compared with 81% of bone metastasis samples. Thus, PTHrP and its receptor are expressed more frequently in bone metastases than primary tumors. However, the functional relevance of this pattern of receptor expression in bone-disseminated tumor cells is still unclear since *in vitro* data indicate that in ER^+ breast cancer cells, activation of PTH1R/cAMP signaling does not regulate dormancy gene expression (95). Nevertheless, while expression of the receptor may not regulate dormancy in the bone, these clinical data

still support the understanding that PTHrP expression by bone disseminated tumor cells is critical to their ability to establish metastatic colonies and possibly promote exit from dormancy. Other studies have also confirmed a positive association between PTHrP expression in primary breast tumors and bone metastasis as well as shortened overall survival (151, 152).

Though in a different metastatic site, a recent study on early stage triple negative breast cancer (TNBC) found that PTHrP expression is significantly correlated with decreased central nervous system (CNS)-progression free survival (153). These findings, if validated in other large cohorts of early-stage, newly diagnosed TNBC patients, would raise the hypothesis that monitoring PTHrP expression in TNBC patients could detect the initial stages of CNS metastasis and identify individuals with recurrent tumors much earlier than conventional detection techniques. Interestingly, this study did not identify a statistically significant relationship between PTHrP expression and the incidence of bone metastasis. It is important to note that only specimens from patients with early stage TNBC without evidence of metastasis at presentation or multiple primary malignancies were analyzed. Thus, examination of patients with later staged cancer may also reveal a significant association between PTHrP expression and bone metastasis in TNBC. This highlights the importance of examining patients with all subtypes of breast cancer and stages of disease progression when investigating PTHrP as a prognostic factor for metastasis and late recurrence.

Clinical evidence also exists suggesting a role for PTHrP in tumor growth and metastasis in other tumor types. In prostate cancer, PTHrP expression varies depending on the cancer stage, with expression detected in 33% of benign prostate hyperplasias, 87% of well-differentiated tumors and 100% of poorly differentiated and metastatic tumors (154). Other studies have similarly found that PTHrP is expressed in prostatic bone metastases (155). Here it seems that a progressive gain of PTHrP in disease progression is associated with tumorigenesis and distant metastasis. In a study of patients with early-stage lung adenocarcinoma, positive staining for PTHrP (1–34) is associated with worse overall survival and metastasis-free survival, independent of tumor stage (156). Survival is even more dramatically reduced in patients with tumors co-expressing high levels of N-terminal PTHrP and PTH1R. Taken together, these observations would suggest that paracrine/autocrine mechanisms involving PTHrP may drive tumor progression in lung cancer.

AUTHORS PERSPECTIVES ON PTHrP AS A PROGNOSTIC FACTOR AND DORMANCY REGULATOR

Given the conflicting data from both preclinical and clinical studies, a general consensus has not yet been reached regarding the role of PTHrP in tumorigenesis, metastasis, and tumor dormancy. However, there are numerous factors to consider when reconciling these findings. Stage of disease progression is critically important in this discussion. In general, the clinical data suggest that early in tumorigenesis at the primary site, PTHrP inhibits cancer growth and progression since its expression is associated with improved survival and decreased metastasis in patients with various tumor types (142, 146, 148, 153) (**Figure 1**). In these cases, tumor cell autonomous actions of PTHrP to alter cell proliferation may account for these findings (68). Late in disease progression, after dissemination to the bone marrow, the growth of surviving tumor cells is driven by increased PTHrP production to stimulate osteoclast-mediated bone resorption, which releases pro-tumorigenic factors that further drive tumor growth and additional PTHrP secretion (17, 18) (**Figure 2**). These paracrine actions of PTHrP mediated by PTH1R signaling in osteoblasts are necessary for bone metastasis growth and would explain clinical findings that PTHrP is associated with reduced disease-free survival and metastasis formation (151, 152). Lastly, the preclinical data clearly indicate that increased PTHrP expression drives breast tumor cells out of their quiescent state (28, 87, 94) *via* a mechanism independent of canonical PTH1R activation. Again, later in disease progression after long latency periods, increased PTHrP expression would favor exit from tumor dormancy in the bone and likely other metastatic sites (**Figure 1**). This hypothesis is supported by preclinical findings that PTHrP downregulates pro-dormancy gene expression (28), promotes proliferation, and inhibits apoptosis (61, 104) which are two key cellular responses that must be carefully balanced to regulate tumor dormancy.

As noted earlier in this review, PTHrP is a molecule with multiple biologically active domains that control its autocrine/paracrine and intracrine actions. Each of these individual actions must be considered when interpreting data on PTHrP as a dormancy regulator and prognostic factor. Preclinical studies have directly demonstrated that manipulating the expression of different PTHrP isoforms elicits markedly different biological responses. A striking example of this comes from Deftos et al. (104) where mice injected with dormant prostate cancer cells expressing the full-length PTHrP (1–173) molecule developed more extensive bone lesions than those injected with PTHrP (1–87) which lacks the full NLS, osteostatin region, and critical mitogen regulatory sequences contained in the carboxy terminus. Findings such as these can be accounted for by multiple factors. There are likely important functional elements in the region of PTHrP spanning amino acids 88–173 that uniquely promote tumor progression in bone but have not been fully elucidated. Furthermore, truncated forms of PTHrP may also assume different tertiary structures which alter binding to or interactions with other proteins that may drastically influence tumor cell behavior. Preclinical studies to further elucidate the biological activity of each PTHrP domain will be critically important to understanding the complexity of the molecule's effects in tumor development.

In interpreting findings from clinical studies on survival and prognosis in human patients, a third factor to consider is the epitope used to define positive and negative expression, as nearly all of these analyses utilize immunohistochemistry to detect PTHrP. For instance, in their work on NSCLC, Montgrain et al. (146) specifically investigated PTHrP (1–34) expression while Monego et al. (156) probed for PTHrP (109–141) and found opposite effects with regards to PTHrP as a prognostic indicator.

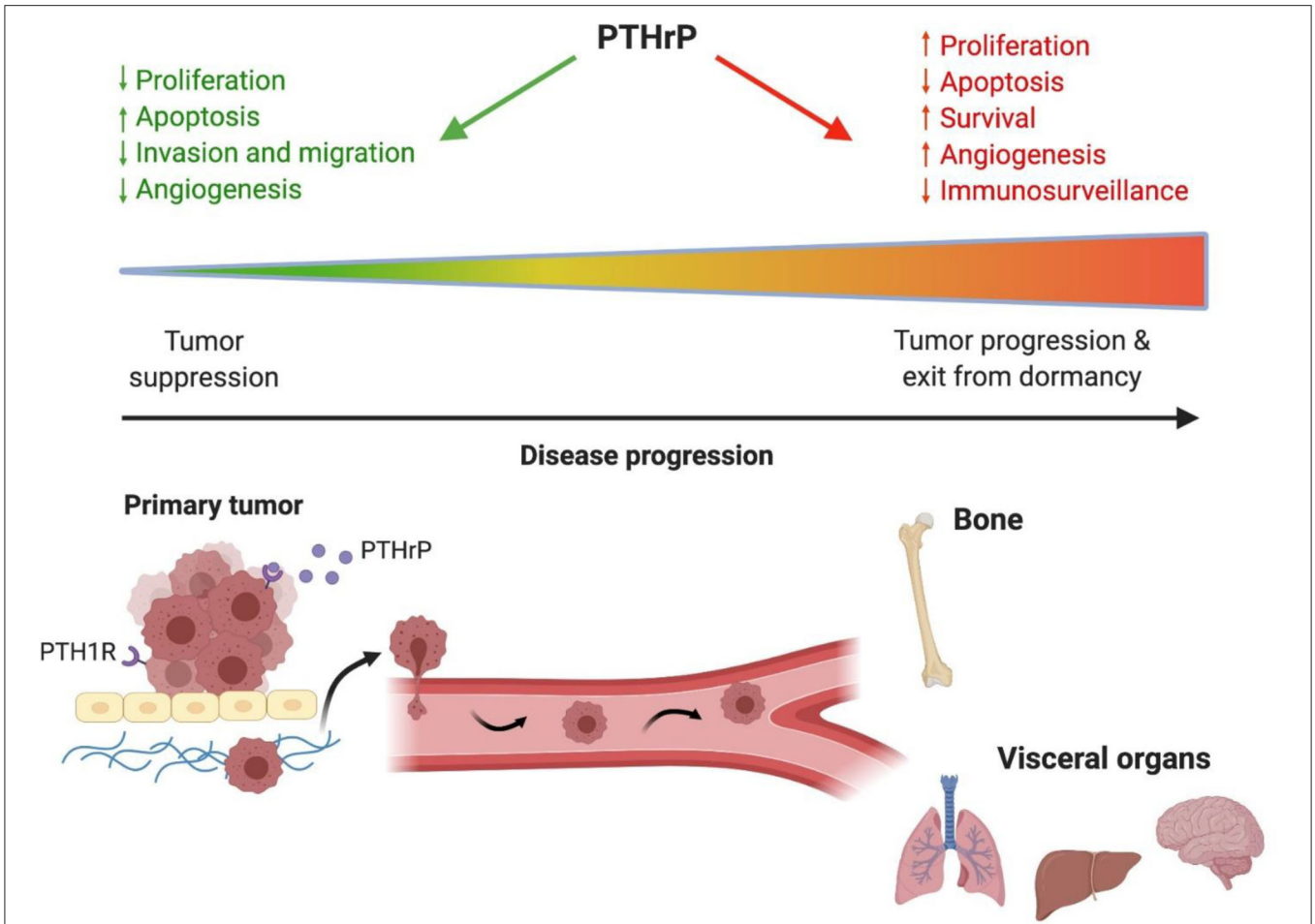

FIGURE 1 | PTHrP has different actions throughout cancer progression. Early in tumorigenesis PTHrP is protective against tumor formation in the primary site by decreasing proliferation, promoting apoptosis, decreasing angiogenesis and reducing tumor cell invasion and migration. Late in disease progression when tumor cells disseminate to distant sites, PTHrP promotes tumor progression and exit from dormancy by stimulating proliferation and angiogenesis while reducing apoptosis and immunosurveillance. These actions in advanced stages of disease contribute to poor patient outcomes and reduced survival. PTHrP, parathyroid hormone-related protein.

Again, amino-terminal and carboxy-terminal PTHrP regions are known to induce disparate biological effects depending on the cell type and activation of autocrine/paracrine vs. intracrine signaling. Due to posttranslational proteolytic processing, the mature PTHrP molecule can also give rise to multiple peptides with different biological activities. Fragments encompassing the amino terminal region (residues 1–36), mid-molecule regions (38–94), (38–95), and (38–101), as well as the carboxy terminal (107–139) have been detected. Multiple peptide fragments have even been isolated from plasma (157) and urine of patients with HHM (158). Thus, antibody selection is important to consider when drawing conclusions from clinical studies relying on the immunohistochemistry to analyze expression of PTHrP and any of its cleavage products as a prognostic factor.

PTHrP AS A THERAPEUTIC TARGET

Numerous studies have provided convincing evidence that PTHrP promotes tumor progression, and late recurrence by pushing tumor cells out of dormancy, resulting in poor patient survival. Thus, PTHrP would seem to be a promising therapeutic target for treating advanced human cancers. Several animal studies have demonstrated reduced distant metastasis to bone with PTHrP small molecule inhibitors (159) and neutralizing antibodies (68, 160, 161); however, human clinical data are lacking. Furthermore, there are several limitations in our current understanding of the biological activity of PTHrP that greatly complicate the development of safe and efficacious anti-PTHrP therapies at this time. PTHrP is an incredibly complex peptide with multiple distinct domains that can each influence its actions as an endocrine, paracrine, autocrine and intracrine signaling molecule. This coupled with the fact that its different isoforms and fragments can elicit diverse cellular responses could result in PTHrP targeting therapies that inadvertently promote tumor growth and recurrence if used in the wrong patient population or stage of disease progression. This is especially true in breast cancer, where preclinical and clinical data suggest that PTHrP inhibits early tumor progression, but promotes distant metastasis

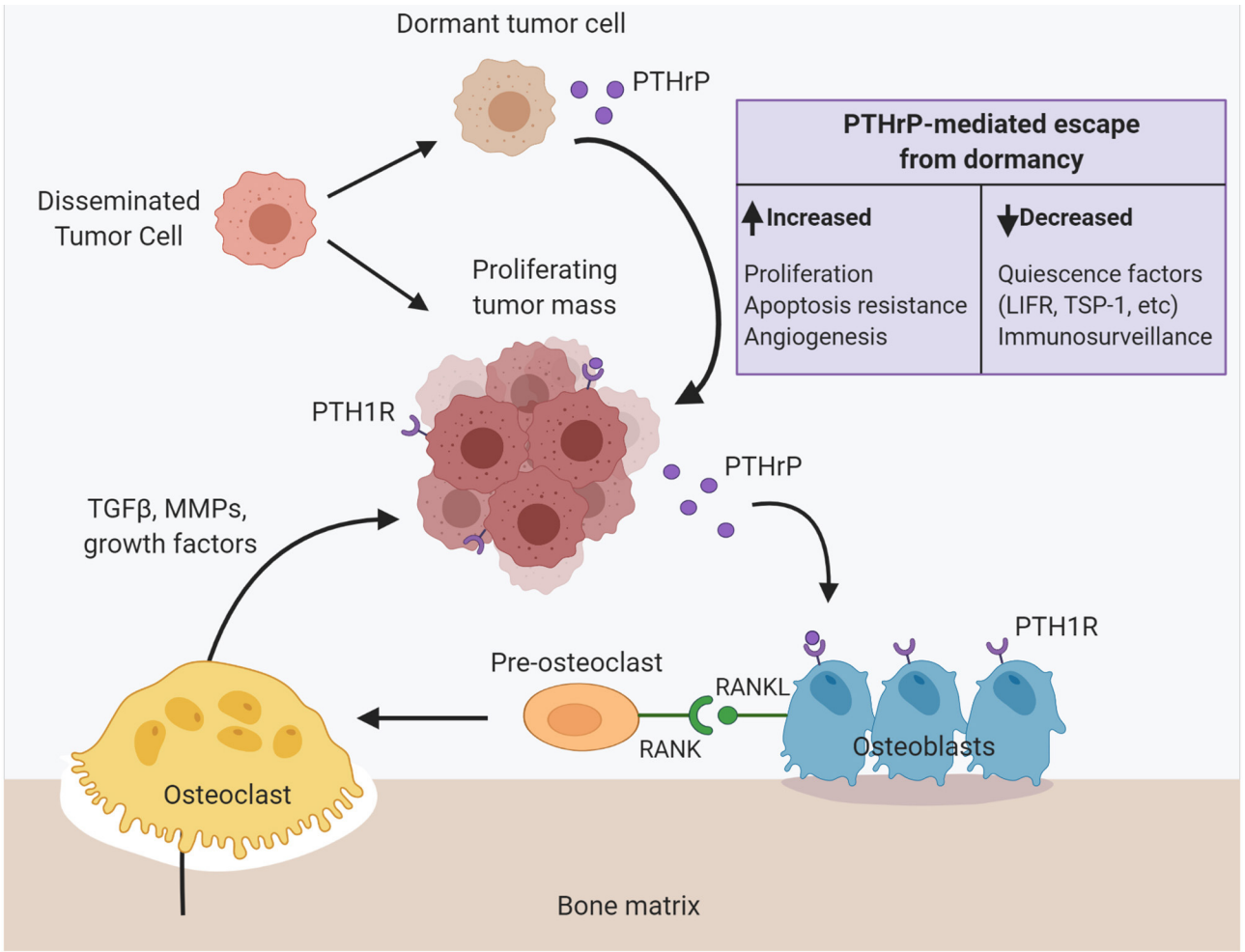

FIGURE 2 | PTHrP dictates disseminated tumor cell fate in the bone to drive metastasis formation. Upon dissemination to the bone, surviving tumor cells can proliferate into a micrometastasis. Tumor cell secretion of PTHrP signals through the PTH1 receptor (PTH1R) on osteoblast lineage cells to stimulate RANKL production and osteoclastogenesis. Osteoclast-mediated resorption releases pro-tumorigenic factors from the bone matrix such as TGF-β, matrix metalloproteinases and other growth factors that further fuel tumor cell colonization, proliferation, and PTHrP production. Alternatively, disseminated tumor cells instead enter a prolonged dormant state. PTHrP drives tumor cell escape from dormancy and metastatic outgrowth via multiple mechanisms: (1) increased proliferation, (2) apoptosis resistance, (3) increased angiogenesis, (4) decreased immunosurveillance and myeloid-derived suppressor cell recruitment, (5) decreased expression of known quiescence factors (e.g., LIFR). PTHrP, parathyroid hormone-related protein; PTH1R, parathyroid hormone-related protein type 1 receptor; RANKL, receptor activator of nuclear factor–kappa B ($NF_{\kappa}B$) ligand; LIFR, leukemia inhibitory factor receptor (LIFR); TSP-1, thrombospondin-1.

in advanced stages of disease (162). Studies fully defining PTHrP's role in different stages of cancer and in tumor dormancy are needed in order to identify the appropriate therapeutic window for targeting PTHrP.

In addition to direct PTHrP inhibition, alternative approaches including targeting upstream regulators of the peptide's expression have been explored. As discussed previously (20), Wnt signaling drives PTHrP expression in highly osteolytic cancer cells and thus presents a potential therapy to prevent tumor-induced bone destruction and metastatic outgrowth. However, there are challenges to targeting Wnt therapeutically due to deleterious off-target effects since signaling is critical during normal development and tissue homoestasis, especially bone formation (163–165). However, the anti-tumor activity of Wnt inhibitors has been investigated and shown varying efficacy, primarily in preclinical gastrointestinal cancer models (166, 167). There are also numerous ongoing clinical trials investigating inhibitors of the Wnt pathway in multiple other solid tumor types [NCT01351103, NCT03901950, NCT02675946, NCT03447470, NCT03395080]. In recent years, more cancer cell-specific molecular targets such as vacuolar-ATPase (v-ATPase) have been explored in the development of Wnt signaling inhibitors (168, 169). Bafilomycin and concanamycin, which directly bind to and inhibit v-ATPase, markedly inhibit Wnt/β-catenin signaling in colorectal cancer cells *in vitro* and reduce tumor cell proliferation *in vivo* without

significant toxicity (168). Selective inhibitors of Porcupine (PORCN), an acyltransferase that catalyzes post-translational modification and activation of WNT ligands, have also shown promising anti-tumor activity *in vivo*, while sparing WNT-dependent tissues (170, 171). While inhibiting the Wnt pathway may be an effective therapy to decrease PTHrP expression for the treatment of metastatic cancers, more extensive investigation is needed to identify the most selective inhibitors and safest therapeutic window.

Alternative upstream targets include TGF-β which upregulates expression of Gli2 and in turn increases tumor secretion of PTHrP (172, 173). Gli2 repression significantly reduces tumor-induced bone destruction mediated by TGF-β signaling in human breast cancer MDA-MB-231 cells (172). Inhibitors against TGF-β and GLI proteins have been evaluated in clinical trials as anti-cancer therapy (174) [clinicaltrials.gov]. Another study demonstrated that the EGF receptor promotes PTHrP production, since treatment with erlotinib, an EGF receptor tyrosine kinase inhibitor, suppresses PTHrP expression in non-small cell lung cancer cells and reduces osteolysis (175). Other EGF receptor tyrosine kinase inhibitors including gefitinib also reduce PTHrP levels (176). Lastly, targeting downstream effectors of PTHrP may also provide an efficacious strategy. For instance, as mentioned previously, PTHrP (1–139) overexpression in MCF7 cells also represses expression and downstream signaling of LIFR, a known breast tumor suppressor and dormancy factor in the bone (28). Consequently, LIFR downregulation promotes human MCF7 breast cancer cell emergence from dormancy in the bone. Treatment with the histone deacetylase inhibitor valproic acid subsequently increases LIFR expression in human MCF7 breast cancer cells *in vitro*, suggesting that targeting LIFR, a downstream factor in PTHrP signaling, may effectively maintain tumor cells in a dormant state to prevent metastatic outgrowth. Multiple strategies should therefore be considered in order to develop the most selective and effective PTHrP targeting therapies.

CONCLUSIONS

PTHrP is a unique multifunctional protein with diverse effects on tumor cell behavior mediated by its different biological domains and isoforms that arise from posttranslational processing. Overall, preclinical and clinical studies suggest that PTHrP inhibits tumor progression in early stages of disease while it functions in the opposite manner to promote tumor development and metastasis formation in advanced cancers, resulting in diminished survival in patients. This is especially true in the bone, a common site of metastasis, where PTHrP-mediated osteolysis is critical for tumor cells to establish as colonies and grow. Furthermore, while the studies are still limited, an important role for PTHrP in promoting tumor emergence from a dormant state is an emerging area of interest. Despite its complexity, more studies that fully uncover the unique biological activities of PTHrP and its domains that regulate its endocrine, autocrine, paracrine, and intracrine signaling could uncover numerous additional targets to explore as anticancer therapeutics.

AUTHOR CONTRIBUTIONS

CE drafted the manuscript. CE and RJ edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Innovative Approaches in the Battle Against Cancer Recurrence: Novel Strategies to Combat Dormant Disseminated Tumor Cells

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Cancer recurrence remains a great fear for many cancer survivors following their initial, apparently successful, therapy. Despite significant improvement in the overall survival of many types of cancer, metastasis accounts for ~90% of all cancer mortality. There is a growing understanding that future therapeutic practices must accommodate this unmet medical need in preventing metastatic recurrence. Accumulating evidence supports dormant disseminated tumor cells (DTCs) as a source of cancer recurrence and recognizes the need for novel strategies to target these tumor cells. This review presents strategies to target dormant quiescent DTCs that reside at secondary sites. These strategies aim to prevent recurrence by maintaining dormant DTCs at bay, or eradicating them. Various approaches are presented, including: reinforcing the niche where dormant DTCs reside in order to keep dormant DTCs at bay; promoting cell intrinsic mechanisms to induce dormancy; preventing the engagement of dormant DTCs with their supportive niche in order to prevent their reactivation; targeting cell-intrinsic mechanisms mediating long-term survival of dormant DTCs; sensitizing dormant DTCs to chemotherapy treatments; and, inhibiting the immune evasion of dormant DTCs, leading to their demise. Various therapeutic approaches, some of which utilize drugs that are already approved, or have been tested in clinical trials and may be considered for repurposing, will be discussed. In addition, clinical evidence for the presence of dormant DTCs will be reviewed, along with potential prognostic biomarkers to enable the identification and stratification of patients who are at high risk of recurrence, and who could benefit from novel dormant DTCs targeting therapies. Finally, we will address the shortcomings of current trial designs for determining activity against dormant DTCs and provide novel approaches.

Keywords: tumor dormancy, metastasis, disseminated tumor cells, cancer recurrence, tumor microenvironment, immune evasion and clinical trials

INTRODUCTION

Recent years have seen great strides in the treatment of primary tumors, as well as in treating overt metastatic tumors. Nonetheless, the main cause of mortality of cancer patients remains metastasis and recurrence. Metastasis, the spread of tumor cells from the primary site to secondary organs accounts for 67-90% of all cancer mortality (1, 2). Despite significant improvement in the overall survival of many types of cancer due to earlier detection and newer therapies, recurrence years and decades after curative surgery and standard of care chemotherapy and targeted therapy (3–5) still looms as the major unmet medical need.

Some cancers are more notorious than others for delayed recurrence. These highly recurrent tumors include kidney cancer, acute myeloid leukemia (AML), non-small cell lung cancer (NSCLC), melanoma, prostate cancer, ovarian cancer, breast cancer and osteosarcoma. Kidney cancer and AML exhibit low single/double-digit recurrence rate (6–8), while many other cancers have a very high rate of locoregional and distant recurrence despite treatment with standard of care (**Table 1**).

Lung cancer is the leading cause of cancer-related death with NSCLC accounting for ~90% of new cases (21). Even when curative surgery is performed, 30-50% of NSCLC patients develop locoregional or distant recurrence (22, 23). Melanoma can recur in 50% of the patients (20) sometimes a decade or more following removal of the primary tumor (24). Prostate cancer, which is a slow growing tumor, nonetheless shows biochemical recurrence (increasing PSA) even in low risk patients beginning some 4 years following therapy with curative intent in some 25% of patients (12). In ovarian cancer, an estimated 85% of patients who achieve full remission after initial treatment (surgery and adjuvant chemotherapy) have a recurrence, with median survival between 12-24 months after recurrence (17, 25). Breast cancer is the most common form of cancer in US women after skin cancer, with over 275,000 cases estimated for 2020. Much progress has been made in the treatment of primary breast cancer, particularly when there is a known mutation or overexpression that can be directly targeted (e.g. PI3K, CDK4/6, PARP, PD-L1) or with hormone receptor-positive disease (e.g. ER+, PR+, HER2+) (26). Even with the wealth of treatment options, around 30% of all breast cancer patients with no detectable disease post-treatment present with recurrence on follow-up (9). Osteosarcoma (OS) is the most common type of primary bone tumor accounting for 30-80% of skeletal sarcomas, and it occurs primarily in adolescence (27). Even with aggressive treatment including limb-salvage surgery and chemotherapy, the rate of recurrence in patients presenting with non-metastatic OS is 30-40% (15, 16). Taken together, despite major advances in the treatment of primary tumors, almost all cancer-related mortality is due to recurrence and metastasis and many of the most common cancers have a significant propensity for delayed recurrence (28).

While metastasis and recurrence are the main cause of mortality in cancer patients, the mechanisms underlying metastatic recurrence years and decades after initial treatments are just beginning to unravel. Recurrence due to metastatic spread begins as a multi-step process that can take months or years until it becomes detectable. Recent studies have shown that although the probability of metastasis increases with the size of the primary tumor, cancer cells nonetheless leave primary tumors early (29–32) and settle in distant tissues to become disseminated tumor cells (DTCs).

Once cancer cells arrive at their new and foreign microenvironment ('non-permissive soil') they face several fates. The majority of them will undergo apoptosis and thus will meet their demise. Those that successfully launch adaptive and survival programs will enter a dormant state. Some dormant DTCs may reside as single solitary quiescent cells (cellular dormancy) and/or as small clusters of quiescent cells. Others may reside as small indolent micrometastases where cellular proliferation is balanced by apoptosis (33–36). These indolent micrometastases remain dormant due to either lack of angiogenic signals (angiogenic dormancy) that promote recruitment of the vasculature needed to nourish the micrometastatic tumor (35, 36) and/or involvement of the adaptive immune system (immune-mediated dormancy) (37). To date, there are no imaging moieties to detect dormant quiescent and indolent micrometastases in patients. Furthermore, due to their quiescence, these dormant DTCs are resistant to classical anticancer therapy that relies on rapidly dividing cells to exert their effect. Therefore, dormant DTCs linger in the body as ticking time bombs and eliminating or keeping such cells at bay may prevent deadly metastatic relapse.

Cell-intrinsic mechanisms governing DTC dormancy and escape from dormancy are influenced by signals arising at their

TABLE 1 | Estimated Recurrence Rate of Various Cancers.

Cancer	Recurrence Rate
Breast	30% distant recurrence (9, 10)
Glioblastoma	~100% (11)
Prostate	20-30% (12)
Leukemia, childhood AML	9-29% (6)
NSCLC	30-50% locoregional or distant recurrence (13, 14)
Osteosarcoma	30-40% (15, 16)
Ovarian	85% (17)
Pancreatic	36% within 1 year of curative surgery 38% local recurrence & 46% distant metastasis after adjuvant chemotherapy (18, 19).
Melanoma	50% of all patients treated for melanoma will have a recurrence. Of these recurrences, ~50% will be in the regional lymph nodes, 20% will be local recurrences, and 30% will arise at distant sites (20)

foreign niche. Accumulating evidence in the literature attributes dormancy and survival of residing DTCs and their reactivation to the intricate cross- talk with their 'non-permissive' or their 'permissive' niche, respectively (38–43). Hence, we can postulate that by manipulating the microenvironment and/or cell-intrinsic mechanisms we may either be able to eradicate dormant DTCs, maintain dormant DTCs at bay, or prevent their transition to overt metastases.

This review will focus on potential strategies, mechanisms and drugs to be considered for targeting quiescent dormant DTCs by manipulating their microenvironment and or their cell-intrinsic mechanisms. We will initially present clinical evidence for the presence of dormant DTCs. Potential biomarkers to enable the identification and stratification of patients who are at high risk of recurrence will also be discussed. Finally, we will address the shortcoming of current clinical trial designs for demonstrating activity against dormant DTCs, either quiescent or indolent, and provide novel trial designs.

CLINICAL EVIDENCE OF TUMOR DORMANCY AND RECURRENCE

Demonstrating the presence of dormant DTCs and their subsequent progression in clinical settings has been challenging. However, advances in detection have provided new information and a growing body of evidence in the clinic to support the idea of early dissemination of tumor cells from the primary site, followed by subsequent dormancy and late recurrence.

One clinical example that supports tumor dormancy is late-stage recurrence. Recurrence of a tumor after more than 5-years remission is in line with an initial dormancy period followed by reactivation and outgrowth. In support of this idea, it was found that breast cancer patients who do not have detectable disease can have circulating tumor cells (CTCs) found in their blood 20+ years after initial treatment (44). The inability of tumor cells to survive decades in the blood supports the hypothesis that these cells are being shed from undetectable DTC populations that have remained dormant for years. Minimal residual disease, or tumor cells that remain in the body after initial treatment, is typically undetectable at the primary site but can be found in the circulation, bone marrow or other organs prone to recurrence (e.g. lungs, liver) (45). Even before late-stage recurrence, these cells can be identified and characterized from blood or bone marrow and have been found to upregulate programs that promote dormancy, survival and progression. One feature observed in bone marrow-resident DTCs is a decrease in proliferation markers Ki67 and proliferating cell nuclear antigen (PCNA), which support the idea that these cells are quiescent and thus less susceptible to cytotoxic chemotherapies (29, 45). Additionally, multiple pathways implicated in dormancy and recurrence in laboratory models are overexpressed in recurrent tumors. In stomach cancer, urokinase-type plasminogen activator receptor (uPAR) was upregulated in patients whose cancer recurred, while low levels of uPAR correlated with longer disease-free periods and survival (46). This same trend was observed with HER2/ERBB2 overexpression in disseminated breast cancer tissue correlating with worse outcomes (47).

Another clinical observation in support of early metastasis and subsequent dormancy can be found in cases of unknown-primary carcinoma (UPC). UPC accounts for ~5% of metastatic cancer cases, and while some cases are later identified after more thorough evaluation, 30% of patients never have a primary site identified (48). These unexplained cases of UPC are believed to have formed from DTCs from a primary tumor that regressed and could no longer be observed (Riethmüller and Klein 2001). Detection of UPC in cases where the primary site is never determined highlight the lack of clinical understanding for tumor cell dissemination.

One of the best pieces of evidence for dormant DTCs that can recur after a long period of quiescence is seen in cases where patients develop tumors after organ transplantation. Accidental transmission of tumors from tissue transplants derived from seemingly cancer-free cadavers was first reported in the case of kidney transplants (48). In one of the earliest reported instances, metastatic squamous cell carcinoma occurred in a patient 8 months after a kidney transplant from a donor later found to have larynx carcinoma (Tissue Transplantation Still Vexes 1965). Another instance of kidney transplant-related cancer saw the patient remain disease-free for 3 years before being diagnosed with metastatic liver cancer. Surprisingly, one study found that of 164 patients receiving organ donations from donors eventually diagnosed with cancer, 44% developed tumors with the majority of those cases related to the tumors of the original donors (49). In a later study, a heart donor was diagnosed with prostate cancer post-mortem, and 10 months after transplant the recipient was diagnosed with multiple metastatic lesions in the spine, sacrum and ribs. Genetic analysis of the lesions indisputably matched with the donor's prostate and kidney, providing evidence at the molecular level that the tumor was derived from quiescent prostate DTCs in the donor's heart (50). All of this clinical evidence provides direct support that tumor dissemination can be an early event and tumor dormancy allows these cells to evade therapy and the immune system and detection for many years, even decades, before leading to recurrence.

PUTTING DTCs UNDER THE DORMANCY SPELL

Clinical data demonstrate the presence and persistence of dormant DTCs years and even decades after treatment. In some cases, these DTCs will remain dormant without relapsing. Hence, unraveling the mechanisms responsible for their long term 'hibernation' may set the premise to develop novel therapeutic strategies to prevent cancer from recurring by keeping them dormant indefinitely.

Reinforcing the Dormant Niche

Several restrictive signals have been described in the bone marrow (BM) and lung that maintain DTCs originating from

breast, prostate, head and neck squamous carcinoma and multiple myeloma cells in their quiescent state (**Figure 1**). Therefore, the BM is seen as a sanctuary site for DTCs. Growth arrest-specific 6 (GAS6) (51), Wnt5α (52), leukemia inhibitory factor (LIF) (53) and TGF-β family members such as bone morphogenic protein 7 (BMP7) (54) and transforming growth factor beta 2 (TGFβ2) (55, 56) were shown to exert quiescence of DTCs at the BM niche, whereas, bone morphogenic protein 4 (BMP4) was shown to promote tumor dormancy in the lung (57). Hence, theoretically we can postulate that DTCs can be maintained quiescent for prolonged periods of time by introducing the restrictive mediators that constitute the dormant niche (42).

SPARC, also known as osteonectin, was shown to regulate tumor dormancy of prostate cancer cells by promoting the expression of BMP7 in BM stromal cells. SPARC was shown to be epigenetically silenced in aggressive cells by promoter methylation whereas treatment of prostate cancer cells with the DNA demethylating agent 5-azacytidine (5-AZA) or with the COX2 inhibitor NS398 could restore SPARC expression in malignant prostate cancer cells. This in turn promoted BMP7 expression in BM stromal cells leading to dormancy of prostate cancer cells (58). Hence, reinstating SPARC expression in bone DTCs by treatment with either 5-AZA or with COX2 inhibitor NS398 may offer a therapeutic window to treat recurrent prostate cancer disease (**Figure 1**).

Another component that may be reinforced is thrombospondin-1 (TSP1), which is found in the suppressive BM and the lung niches. TSP1 secreted by stable microvasculature or by recruited BM-derived myeloid cells was shown to induce tumor dormancy of breast cancer cells at the perivascular niche (PVN) in the BM (59) and prevent metastatic outgrowth of breast and prostate cancer cells in the lungs (60). Given that TSP1 is a large protein, it is not feasible to consider it as a potential treatment. However, TSP1 can be induced by prosaposin. Indeed, administration of the TSP1 mimetic peptide prosaposin (DWLPK) was shown previously to induce TSP1 in BM-derived myeloid cells recruited to the lungs. The latter in turn assembled a metastasis-suppressive microenvironment (60) (**Figure 1**).

Hence, the approach of inducing natural factors of the suppressive niche such as TSP1 and or SPARC is very appealing. However, this approach will require continuous treatment to ensure indefinite quiescence of the residing dormant DTCs and thus may lead to potential toxicities. Furthermore, TSP1 has been also reported to exert opposing effects which can be also attributed to multiple receptors that mediate TSP1 signaling (61). Likewise, SPARC was reported to have context and tumor dependent impacts on tumor progression (62). Therefore, we must consider TSP1/SPARC's multiple effects along with the type of tumor and stage of the disease if we want to consider it as a future preventive treatment for cancer recurrence. Another important aspect that should be taken into account when striving to reinforce the dormant niche with TSP1 or other suppressive constituents such as TGFβ family members, is the role they also play in the immune evasion of DTCs (61, 63).

Therefore, it may be more practical to induce common cell-intrinsic mechanisms converging from the different microenvironmental cues comprising the dormant niches.

Promoting Cell-Intrinsic Mechanisms to Induce Tumor Dormancy

Pioneering work by Aguirre-Ghiso and colleagues identified p38-MAPK (mitogen-activated protein kinase) induction vs. reduction in ERK (extracellular signal-regulated kinase)-MAPK

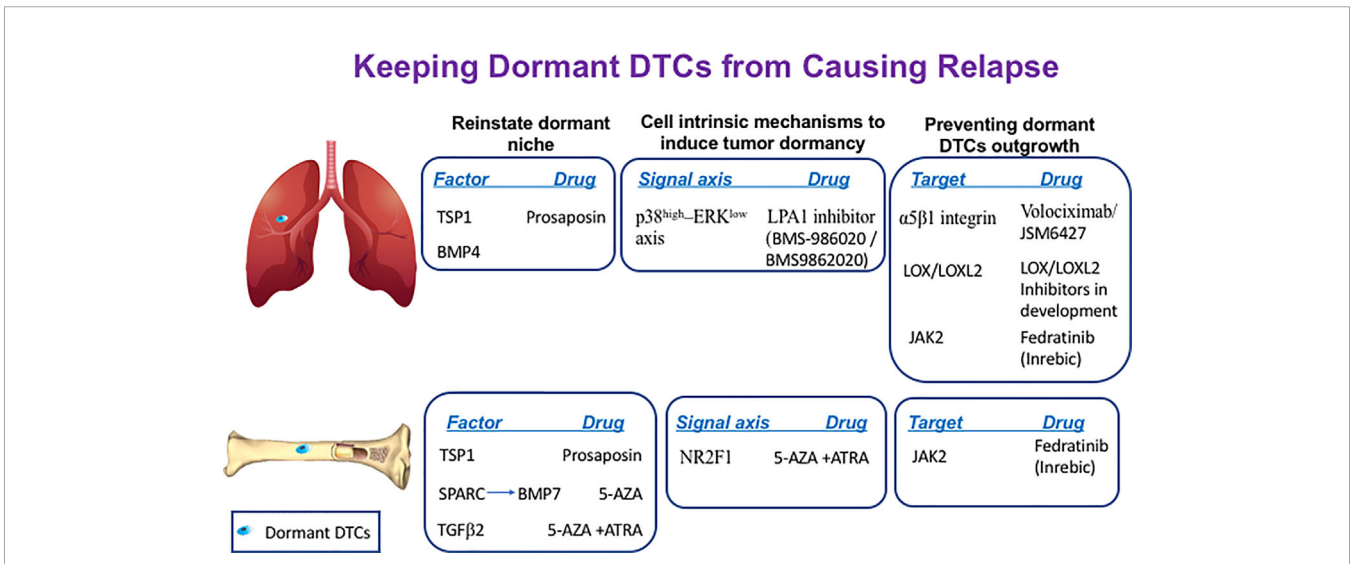

FIGURE 1 | Keeping dormant DTCs from causing recurrence. The following scheme illustrates the different sites where dormant DTCs reside and potential factors, signaling axis or targets that can be manipulated by the indicated drugs to maintain their long-term quiescence by either reinstating the dormant niche, inducing cell-intrinsic dormancy mechanisms and/or preventing dormant DTCs engagement with their 'permissive niche'.

signaling ($p38^{high}$–ERK^{low} signaling axis) as hallmark of tumor dormancy in several types of tumors (35, 64–66). Interestingly, Debio-0719, an inhibitor of lysophosphatidic acid receptor 1 (LPA1), was shown to induce tumor dormancy of triple-negative breast cancer (TNBC) cells at distant organs by inducing the $p38^{high}$–ERK^{low} signaling axis (67). Though this inhibitor is still at the preclinical stage, some other inhibitors of LPA1 are currently being tested in clinical trials for fibrosis. These inhibitors include LPA1 inhibitor BMS986202 (previously AM152) and BMS-986020 [reviewed in (68)]. Therefore, potential use of these inhibitors in TNBCs patients as means to maintain residual disease at halt should be considered for future investigations (**Figure 1**).

Orphan nuclear receptor NR2F1 was shown to be a critical node for dormancy induction in head and neck squamous cell carcinoma (HNSCC) and in DTCs of prostate cancer patients (69). NR2F1 was found to control tumor cell dormancy *via* SOX9 and RARβ-driven quiescence programs (70). Furthermore, combining 5-AZA with trans-retinoic acid (ATRA), reinstated in part the NR2F1-induced dormancy program in HNSCC (70) and induced TGFβ2. TGFβ2 is a BM-derived factor shown previously to impose dormancy in HNSCC and in prostate cancer cells (55, 56). Hence, this combination can induce both dormancy programs and may also contribute to the formation of dormant niche (**Figure 1**).

Preventing the Reawakening of Dormant DTCs by Targeting Their Crosstalk With Their Supportive Niche

The microenvironment of the metastatic niche (34, 36, 40, 71) and its remodeling (35, 38) plays a fundamental role in dictating the fate of residing dormant DTCs by inducing cell-intrinsic mechanisms culminating in the escape from tumor dormancy (39) (**Figure 1**).

Several reports implicated the role of chronic inflammation (72–74) and/or fibrosis (75, 76) as instigators of DTCs awakening. Fibrosis occurs due to a dysregulated wound healing response. Formation of a fibrotic-like milieu in the lung enriched with type I collagen (Col-I) and fibronectin was part of the tumor 'permissive' microenvironment to support dormant mammary DTCs outgrowth (75). Utilizing a 3D model system to study tumor dormancy (77, 78) it was demonstrated that fibronectin and Col-I induced beta 1 integrin (Intβ1) downstream signaling in dormant mammary cells *via* activation of focal adhesion kinase (FAK) by Src. This activation resulted in downstream activation of ERK, which in turn induced phosphorylation of myosin light chain (MLC) by myosin light chain kinase (MLCK), culminating in F-actin stress fiber organization and transition from quiescence to proliferation. Inhibition of MLCK activation (75, 77) and or Intβ1 expression (75) prevented dormant DTCs outgrowth *in vitro* and *in vivo*. Similarly, sustained lung inflammation caused by tobacco smoke exposure or nasal instillation of lipopolysaccharide (LPS) induced the outgrowth of dormant DTCs in the lungs by formation of neutrophil extracellular traps (NETs), which in turn lead to cleavage of laminin-111 by NET-derived elastase and MMP-9 and induction of the Intβ1/Src/FAK/MLCK axis (79). In addition, prostate cancer patient-derived xenograft lines were shown to transition from their dormant state once they engaged with the BM stoma by constitutively activating MLCK (80). Hence, MLCK may serve as potential target to prevent awakening of the dormant DTCs. However, given that MLCK is widely expressed in many normal tissues and in smooth muscle cells, presents a clinical challenge that may require the exploration of other avenues to inhibit MLCK activation in dormant DTCs. One such indirect approach to consider is inhibiting Intβ1 activation.

Notably, several studies highlighted the potential role of Intβ1 activation in regulating the dormant to proliferative switch (81, 82). Previous work reported how the cross talk between Intβ1 and the urokinase receptor can dictate the fate of dormant breast and head and neck cancer cells (83, 84). Intβ1 partners with α subunits to form 12 potential integrin receptors, which bind to extracellular matrix (ECM) molecules such as collagens, laminin, and fibronectin (85).

Indeed, several pre-clinical studies successfully inhibited Intβ1 activity, including α5β1 [reviewed in (86)] which binds the ECM protein fibronectin. In the clinical settings, the anti-α5β1 integrin antibody, volociximab, in combination with carboplatin and paclitaxel demonstrated some encouraging preliminary results in a Phase Ib clinical trial in advanced non-small-cell lung carcinoma (87). Interestingly, JSM6427 a small molecule inhibitor of α5β1 integrin was evaluated in a Phase I clinical trial for the treatment of age-related macular degeneration. It warrants further investigation whether JSM6427 could also be effective in preventing the awakening of dormant DTCs given its mode of action (http://clinicaltrials.gov/ct2/show/NCT00536016) (**Figure 1**). JSM6427 was shown to inhibit attachment of human retinal pigment epithelium cell (RPE) to fibronectin. This in turn promoted quiescence and cortical organization of the cytoskeleton of RPE. Similarly, inhibition of Intβ1 binding to fibronectin prevented the awakening of dormant mammary cancer cells resulting in their cortical F-actin organization reminiscent of the cytoskeletal organization of dormant DTCs (75, 77).

Therefore, if repurposing JSM6427 to treat cancer patients is being considered, the clinical regimen by which this drug will be administered must also be considered. In light of initial studies demonstrating Intβ1 plays a perquisite role in the reactivation of dormant DTCs [reviewed in (88)] while having no significant impact on actively proliferating metastases (75). Hence, these findings should be considered when designing future clinical trials with α5β1 integrin inhibitors. Changing the therapeutic paradigm of cancer therapy to a preventive treatment targeting early dormant DTCs rather than the current strategies aimed at treating patients with already advanced disease should be considered. Moreover, given that fibronectin and Col-I are part of the fibrotic milieu, preventing engagement of residual cells with such a supportive milieu after local surgery seems crucial.

Indeed, surgical trauma induces local and systemic inflammatory responses that can also contribute to the accelerated growth of residual and micrometastatic disease (89–91). Hence, intervention with inhibitors for α5β1 integrin

should be pre-operative and immediately after surgery (post-operative).

Another receptor shown to interact with Col-I at the permissive site is DDR1. Col-I was shown to boost the association of DDR1 with TM4SF1, which, in turn, induced non-canonical signalling through the JAK2/STAT3 axis in dormant breast cancer cells leading to their outgrowth at multiple organ sites (92). Given that development of DDR1 inhibitors are at their infancy, a selective oral JAK2 inhibitor such as fedratinib (Inrebic®), recently approved by the FDA for the treatment of myeloproliferative neoplasm-associated myelofibrosis (93) may be considered as a potential drug to be tested in a preclinical setting (**Figure 1**).

In addition to preventing the engagement of dormant DTCs with their permissive niche and inhibiting cell-intrinsic mechanisms induced by signals arising at this niche, inhibiting the formation of such a permissive niche could represent a viable approach. For instance, inhibiting the cross-linking of Col-I by either lysyl oxidase (LOX) or lysyl oxidase like 2 (LOXL2), could prevent formation of the fibrotic milieu. Indeed, it was shown that LOX and/or LOXL2 inhibition significantly decreased pulmonary metastatic burden (94, 95). Furthermore, LOXL2 was shown recently to exert a cell autonomous role in the emergence of dormant DTCs. A study by Weidenfeld and colleagues demonstrated that LOXL2 expression induced by hypoxia in dormant breast DTCs promoted their epithelial to mesenchymal transition (EMT). This in turn endowed the cells with stem-like properties leading to their escape from tumor dormancy both *in vitro* and *in vivo*, while inhibiting LOXL2 expression prevented their outgrowth (96, 97). Overall, these studies suggest that LOX/LOXL2 may serve as a therapeutic target to prevent the emergence from tumor dormancy to overt metastases (**Figure 1**).

Interestingly, a recent report demonstrated how a systemic inflammatory response induced after surgery promotes the emergence of dormant immunogenic DTCs at distant anatomic sites while, preoperative anti-inflammatory treatment with meloxicam, a nonsteroidal anti-inflammatory drug (NSAID), prevented the outgrowth of DTCs in the lungs (74). Notably, these finding are in line with a retrospective analysis carried out on breast cancer patients who received anti-inflammatory analgesics prior to surgery. These patients exhibited reduced incidence of early metastatic relapse (98, 99).

Overall, the studies presented here emphasize the important role of inflammation and/or fibrosis in dormant DTCs outgrowth and also reinforce the notion that intervention in the perioperative stage and immediately after re-section may be critical to prevent local and/or distant recurrences.

ERADICATING DORMANT DTCs BEFORE THEY AWAKEN

Once DTCs anchor in their new and "non-permissive niche" adaptive cell-intrinsic mechanisms ensure their long-term survival and escape from immune surveillance (41, 42, 100). These hibernating cells resist most traditional and newer targeted agents given their quiescent state and/or their induced senescence–like state (101) or as recently suggested due to the BM perivascular niche (100). Hence, unraveling cell-intrinsic mechanisms responsible for long-term survival of DTCs along with the mechanisms that enable their chemoresistance and immune evasion may open up new approaches to eradicate these dormant DTCs.

Targeting Cell-Intrinsic Mechanisms Mediating Long-Term Survival of DTCs at the Foreign Niche

The mechanisms responsible for the long-term survival of DTCs are just beginning to emerge.

Previously, Src and ERK1/2 activation were shown to be essential for the survival and outgrowth of dormant breast DTCs. By utilizing a 3D model system and *in vivo* model system to study tumor dormancy (75, 77, 78) it was shown that only combined inhibition of ERK1/2 and Src in dormant breast cells culminated in their eradication (102). These findings suggest that combining a Src inhibitor such as saracatinib (AZD0530) with the FDA-approved MEK1/2 inhibitor trametinib may eradicate dormant breast tumor cells before they awaken (**Figure 2**).

The activation of the transcription factor ATF6α was shown to regulate the survival of quiescent squamous carcinoma cells. ATF6α activation induced survival through the up-regulation of Rheb and activation of Akt-independent mTOR signaling (103). Of note, two mTOR inhibitors have been approved by the FDA to treat cancer and several are under clinical investigation as a combination or monotherapy. However, these clinical trials to date are designed to test the drug efficacy in advanced cancer or recurring cancer patients but not as a monotherapy to target dormant DTCs (104) (**Figure 2**).

Another intrinsic mechanism shown to regulate DTCs survival is autophagy (71, 105, 106) (**Figure 2**). Inhibition of autophagy in dormant breast and osteosarcoma cells by hydroxychloroquine promoted apoptosis of the dormant breast DTCs that have colonized the lungs and sensitized dormant osteosarcoma cells to cytotoxic anticancer agents (106, 107). Interestingly, autophagy was recently shown to restrict the outgrowth of micrometastases of several murine models of breast cancer while inhibition of autophagy lead to their outgrowth (108). Hence, inhibition of autophagy might differentially impact quiescent solitary dormant DTCs vs. micrometastases.

Notably, hydroxychloroquine is being widely used in cancer clinical trials in order to re-sensitize cancer cells to conventional therapies. Potential use of this drug as a preventive treatment to eradicate early DTCs may warrant further investigation (109). Currently, a Phase II trial of hydroxychloroquine with everolimus for prevention of recurrent breast cancer is ongoing, as well as an ongoing Phase Ib/II trial of gedatolisib (inhibitor of PI3K/mTOR pathway), hydroxychloroquine or combination of both [reviewed in (110)].

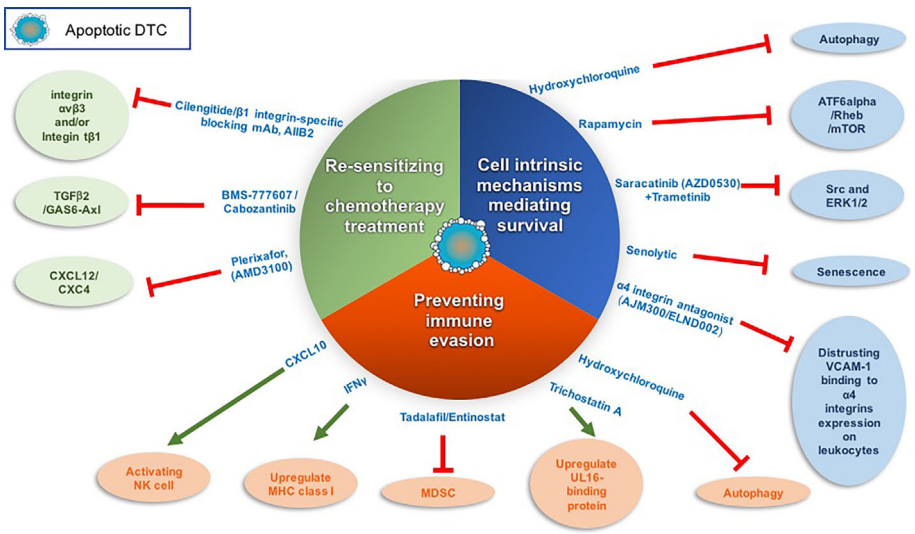

FIGURE 2 | Targeting dormant DTCs for eradication. The following scheme illustrates the different strategies and corresponding drugs that we may utilize to eradicate dormant DTCs. These strategies include inhibiting cell-intrinsic mechanisms of dormant DTCs long-term survival, sensitizing dormant DTCs to chemotherapy treatment and/or preventing dormant DTCs immune evasion. Red line denotes inhibition and green arrow denotes activation.

Aberrant expression of vascular cell adhesion molecule-1 (VCAM-1) on dormant breast DTCs was also found to ensure their survival once they colonize the lungs (**Figure 2**). Specifically, VCAM-1 promoted PI3K/Akt activation and cancer cell survival by its engagement with the counter-receptor α4 integrins expressed on leukocytes. Furthermore, antibodies against α4 integrins blocked pro-survival signals induced by VCAM-1 (111). Therefore, disrupting the interaction between VCAM-1 and α4 integrins may potentially serve as a therapeutic target to promote dormant DTC eradication (112). Such drugs are already in clinical trials for the treatment of relapsing multiple sclerosis (MS) and inflammatory bowel disease (IBD). Currently, an orally active α4 integrin antagonist, AJM300, is under clinical trials in IBD patient [reviewed in (112)].

Notably, senescence of tumor cells that have escaped cytotoxic therapy has been proposed by several groups as another form of cell-intrinsic mechanisms that confer tumor dormancy and survival of circulating tumor cells (CTCs) and/or DTCs residing at the perivascular niche [reviewed in (101)]. This emerging concept warrants further exploration as it may open up an interesting opportunity to target these senescent-like cells with senolytics (small molecule drugs with selective killing of senescent cells) (113, 114) (**Figure 2**). Indeed, several recent papers have demonstrated how senolytic drugs caused cell death of senescence induced cancer cells (115–118).

Overall, these promising studies highlight the importance in further investigating the cell- intrinsic mechanisms governing survival of dormant DTCs.

Sensitizing Dormant DTCs to Chemotherapy Treatments

Signals arising at their foreign niche can induce cell-intrinsic mechanisms governing the survival of dormant DTCs and their escape from cytotoxic therapies. C-X-C motif chemokine ligand 12 (CXCL12) has been reported to constitute aspects of the dormant niche and act as a survival factor. CXCL12 secreted by osteoblasts was shown to induce the survival of disseminated breast tumor cells expressing the CXCR4 receptor by upregulating the Akt pathway *via* c-Src activation (119). Furthermore, the CXCL12/CXCR4 axis was shown also to mediate the localizing and tethering of prostate cancer cells and of breast cancer cells to the BM (120–122) and regulate their growth. Therefore, disrupting the CXCL12/CXCR4 axis may impinge on the survival of dormant breast tumor cells or induce the outgrowth of prostate tumor cells (123). Plerixafor (AMD3100), a CXCR4 antagonist approved by the FDA, was shown in a subcutaneous xenograft mouse model of human prostate carcinoma to dissociate the prostate cancer cells from their sanctuary site (the BM) and thus sensitize them to chemotherapy treatment (124). This approach was demonstrated to be effective also for other cancers, such as leukemia (125–127). Thus, dormant DTCs may be sensitized to chemotherapy by promoting their proliferation making them vulnerable to chemotherapy treatment (**Figure 2**).

F-box/WD repeat-containing protein 7 (FBXW7) was shown to promote quiescence by ubiquitylation and proteasomal degradation of cell cycle promoters. Ablation of FBXW7 in dormant breast cancer cells caused DTCs to exit their quiescent state, which sensitized the cells to paclitaxel treatments in mouse xenograft and allograft models (128).

Recently, the TGFβ2/GAS6-Axl axis (56) was shown to be necessary for the induction of dormancy of prostate cancer cells in the BM. Treatment of dormant multiple myeloma cells with inhibitors to Axl such as BMS-777607 or cabozantinib released the cells from the endosteal niche in the BM, causing their reactivation, thus suggesting these cells could be susceptible to

chemotherapy treatment (129). Indeed, in previous studies in solid tumors, combining chemotherapy treatment with inhibitor of Axl reduced tumor burden (130).

Another protein mediating the engagement of cancer cells to the BM niche is osteopontin. Osteopontin is an ECM protein secreted by osteoblasts in the BM niche and was shown to anchor acute lymphoblastic leukemia (ALL) cells to the BM culminating in their dormancy. Whereas, inhibition of osteopontin promoted ALL escape from tumor dormancy and sensitized them to cell-cycle–dependent Ara-C chemotherapy (131).

Overall, these studies suggest that dissociating dormant DTCs from their 'safe haven' niche can promote the cells to exit their quiescent state and potentially sensitize them to chemotherapy treatments.

Importantly, there are striking similarities between dormant DTCs and quiescent normal hematopoietic stem cells (HSCs), which reside in the BM. These similarities are exhibited by intrinsic mechanisms for survival, quiescence and their mobilization to the BM [reviewed in (43, 132)]. Therefore, when considering a strategy to dissociate dormant DTCs from their sanctuary site in order to sensitize DTCs to cytotoxic chemotherapy treatment it is critical to be sure that this will not facilitate depletion of HSC or cause reawakening of chemoresistant DTCs.

A recent report by Ghajar and his colleagues (100) demonstrated that disrupting the interaction between chemoresistant DTCs with the perivascular niche (PVN) by inhibiting Intβ1 and/or integrin αvβ3 sensitized DTCs to chemotherapy without inducing DTC proliferation (**Figure 2**). However, 22% of mice still succumbed to bone metastases upon combining integrin β1 inhibition with adjuvant therapy in this study.

Hence, there exists a risk of DTCs mobilization from their dormant niche. Therefore, another strategy that we may contemplate to eradicate dormant DTCs is by preventing their immune evasion.

Breaking Off Immune Evasion of Dormant DTCs

A recent report by Malladie and colleagues demonstrated that cancer cells selected from lung and breast cancer cell lines for their competence to establish latent metastasis (dubbed LCC), acquired quiescence with stem-like characteristics by expressing the Wnt inhibitor DKK1. The authors demonstrated that autocrine DKK1 helps disseminated LCC cells enter quiescence. Furthermore, these quiescent cells downregulated natural killer (NK) cell ligands leading to evasion of immune surveillance. Specifically, quiescent LCCs downregulated cell surface UL16-binding protein (ULBP) activators of NK cell-mediated cytotoxicity, as well as receptors for cell death signals. Therefore, quiescent (dormant) LCCs escaped cytotoxic killing by NK cells whereas proliferating LCCs were eradicated by activated NK cells (133). Of note, this study was conducted in immune-compromised mice and hence the role of NK cells in fully immune-competent animals remains to be determined.

Overall, these initial findings may open up in the future a novel therapeutic approach to selectively eradicate dormant DTCs by inhibiting their immune evasion. This will require re-expression of NK ligands in dormant DTCs. Interestingly, the inhibitor of histone deacetylases (HDACs) trichostatin A (TSA) was shown to induce ULBP expression in epithelial cancers (134). Whether TSA can inhibit the immune escape of dormant DTCs remains to be explored (**Figure 2**).

Notably, several studies demonstrated how autophagy in cancer cells can impair the susceptibility of the cancer cells to NK-mediated killing (135). Given that autophagy was shown previously to be launched in dormant DTCs (71, 106), it will be worth exploring whether inhibiting autophagy of dormant DTCs will not only impinge on their direct survival but may also enable cytotoxic killing by NK cells (**Figure 2**).

Another mechanism by which DTCs were shown to evade the immune system is by downregulation of the expression of major histocompatibility complex class I protein (MHC-I), thus evading CD8+ T cell recognition (136). Furthermore, the MHC-I negative phenotype of DTCs in the BM was shown to be associated with poor survival in curatively resected breast cancer patients without distant metastases (137). Similarly, single quiescent DTCs colonizing livers from patients and mice with pancreatic ductal adenocarcinoma (PDAC) were MHC-I-negative and exhibited unresolved endoplasmic reticulum (ER) stress. Notably, once these quiescent DTCs were resolved from their ER stress the cells emerged from their dormant state but also regained their MHC-I expression. Therefore, outgrowth of these cells occurred only when these quiescent DTCs were resolved from their ER stress and the T cell response was disrupted (138).

Hence, upregulating MHC-I may be an attractive approach to reinstate DTCs vulnerability to immune surveillance (**Figure 2**). Notably, epigenetic control mechanisms regulating MHC-I expression have been frequently detected [reviewed in (139)]. Furthermore, interferon γ (IFNγ) was shown by several studies to act as an epigenetic modifier upregulating the expression of antigen-presenting machinery genes such as MHC-I (140). Future studies should be pursued in order to study whether treatment of dormant quiescent PDAC/breast DTCs with IFNγ will induce MHC-I antigen expression and eradication by CD8+ T cells. This kind of approach needs to take into account: i) whether the dormant DTCs with MHC-I downregulation can be sensitized to IFN-γ treatment. Given that approximately 30% of human tumor cells exhibit reduced IFN-γ sensitivity as a result of an impaired expression in the different components of the IFN-γ signaling (139) and ii) the clinical stage and context by which this treatment will be applied. Considering that IFN-γ has pleotropic effects on different stages in tumor progression (141). In addition, IFN-γ also is toxic given systemically, so inducing expression locally is likely to be more successful than systemic administration.

Overexpression of immune checkpoint proteins on dormant tumor cells was also shown to facilitate their immune escape. Dormant tumor cells in the DA1-3b/C3H mouse model of AML evade cytotoxic T-lymphocyte (CTL)-mediated killing because they overexpress PD-L1 (B7-H1) and CD80 (B7-1) (142). PD-L1 binds to receptors on CTLs (PD-1) and thus promotes CTL death and exhaustion. Importantly, this immune evasion was

overridden by activating NK cells with CXCL10 (143). Overall, these findings suggest that reinstating either MHC-I and/or NK cell ligands, as well as inhibiting immune checkpoints proteins on dormant DTCs, may re-sensitize them to cytotoxic killing by T cells and/or NK cells (**Figure 2**).

Myeloid–derived suppressor cells (MDSCs) may also indirectly regulate the survival of dormant DTCs. MDSCs represent a population of special cells of the immune system, which consist of immature macrophages, immature granulocytes, and immature dendritic cells. MDSCs suppress activation of T and NK cells through the production of reactive oxygen species (ROS) and arginase 1 (Arg-1), along with recruitment of other immune suppressive cells such as regulatory T cells [reviewed in (144)]. Accumulating evidence suggests that enrichment and activation of MDSCs correlates with cancer recurrence and poor clinical outcome.

Hence, modulating MDSC immunosuppressive activity may in turn prevent immune evasion of dormant DTCs. Inhibitors of phosphodiesterase-5, sildenafil and tadalafil, were shown to inhibit the immunosuppressive activity of MDSCs in preclinical and clinical studies by the downregulation of inducible nitric oxide synthase (iNOS) and Arg-1 activities (144). Promising results with tadalafil have been reported for head and neck squamous cell carcinoma and melanoma patients (145–147). Entinostat, a class I histone deacetylase inhibitor, was also shown to inhibit the immune suppressive activity of MDSCs [reviewed in (144)]. Importantly, entinostat has been evaluated in Phase I and II trials in patients with advanced malignancies, with a favorable risk–benefit profile [reviewed in (148)].

Overall, several drugs that have already been tested in the clinical setting may be considered for inhibiting the immune evasion of dormant DTCs thus potentially leading to their demise (**Figure 2**).

TARGETING POTENTIAL GATEKEEPERS IN REGULATION OF DORMANT DTCs AND THEIR SUPPORTIVE MICROENVIRONMENT

Current studies which are just beginning to unravel the intricate crosstalk between the residing dormant DTCs and their niche, highlight the complexity and the need to rethink the design of future therapeutic strategies to prevent dormant DTCs from ever emerging. If a common multi-faceted target that will both inhibit dormant DTCs and their niche can be identified, the cross-talk between them could be affected leading to their long-term hibernation or their demise.

Potential Gatekeepers That We May Consider Inhibiting Based on Current Studies Are STAT3 and Reinstating NR2F1 Expression

A potential multifaceted target that has been demonstrated to be a molecular hub in mediating tumor escape from immune surveillance (149) and regulate the outgrowth of dormant DTCs is STAT3. Expansion and immunosuppression of MDSCs is mediated by activation of STAT3 [reviewed in (149)]. Anti-inflammatory M2-like macrophages which also play an important role in metastasis at distant organs [reviewed in (150)] are polarized to the M2 phenotype by STAT3 activation [reviewed in (149)]. Furthermore, in addition to STAT3's role in immune suppression, STAT3 activation was recently shown to also directly mediate dormant DTC outgrowth. DDR1 interaction with Col-I at the permissive niche induced non-canonical signaling converging on activation of STAT3 in dormant breast DTCs, which culminated in their outgrowth at multiple organ sites (92). Notably, in paraffin-embedded tissue microarray sections of human breast cancers there was a significant increase in the expression of phosphorylated STAT3 (pSTAT3) in lung metastases compared to their matched primary tumors and the highest levels of pSTAT3 were present in those that had recurred after a short disease-free interval (92). These results suggest a role of activated STAT3 in metastatic outgrowth of dormant breast DTCs in the lungs. Therefore, targeting STAT3 may prevent both dormant breast DTCs outgrowth at the permissive site in the lungs and the formation of an immune suppressive niche.

Hence, STAT3 may serve as an attractive clinical target given that inhibitors of STAT3 have already entered clinical trials along with newer inhibitors at the preclinical stage [reviewed in (151)]. Moreover, several FDA-approved drugs were shown already to inhibit STAT3 signaling and thus may be repurposed to potentially prevent the outgrowth of dormant breast DTCs (152). However, it is important to keep in mind STAT3's central role in signaling networks and therefore targeting it may lead to toxicity.

Of note, STAT3's role in cancer recurrence is just beginning to unravel, and a recent study in contrast demonstrated that dormant breast DTCs residing in the BM niche remain dormant upon activation of STAT3 *via* the activation of leukemia inhibitory factor (LIF) receptor (53). Furthermore, inhibition of STAT3 led to bone osteolysis resulting in the outgrowth of dormant DTCs (53). Hence, LIFR : STAT3 signaling appears to confer a dormancy phenotype in breast cancer cells disseminated to bone. In addition, STAT3 was shown to be part of a pro-dormancy gene signature for estrogen receptor positive breast tumors (153)

Therefore, further research needs to be conducted in order to clarify the role of STAT3 in dormancy and outgrowth. It may be that the outcome of targeting STAT3 may depend on breast cancer subtype, the site of hibernation of the dormant DTCs and the precise timing of such intervention.

Another gatekeeper that holds great promise is NR2F1. Reinstating NR2F1 expression in the BM may prevent dormant DTCs from awakening by promoting cell-intrinsic dormancy programs in prostate and/or HNSCC cells while also reinstating the dormancy niche in the BM by secretion of BMP7 and TGFβ2 (69, 70). Furthermore, SPARC, which is a target of NR2F1 (58), was shown to regulate tumor dormancy of prostate cancer cells by promoting the expression of BMP7 in BM stromal cells.

Indeed, recent studies demonstrated that combining the epigenetic regulating drug 5-AZA with the differentiation agent trans-retinoic acid (ATRA), reinstated in part NR2F1 expression (70), while 5-AZA by itself reinstated SPARC expression in bone DTCs (58). This combination is now part of an ongoing Phase II clinical trial (https://clinicaltrials.gov/ct2/show/NCT03572387) to study combined 5-AZA and ATRA treatment on top of standard of care in recurrent prostate cancer patients based on rising prostate-specific antigen (PSA) only.

Overall, these studies may open up novel avenues to maintain DTCs dormancy.

POTENTIAL MARKERS THAT WILL ENABLE STRATIFICATION OF PATIENTS AT HIGH RISK FOR RECURRENCE

Once a therapy is developed to either target dormant DTCs for destruction or prevent their emergence from dormancy, the question arises as to which patients are at sufficient risk to warrant a therapy in the absence of overt disease. The decisions by clinicians to treat patients using such therapies will largely depend on the risk/benefit potential for such therapies, as well as healthcare-associated costs. Assuming that novel treatments to prevent recurrence will involve risks and substantial costs, the ability to stratify patients and determine for which patients the treatment is likely to provide benefit would be extremely valuable. This is true for clinical trials and post approval.

Patient prognosis and stratification in order to inform treatment decisions is far from being a simple endeavor. Dormancy and emergence from dormancy are presumably determined by properties of individual dormant DTCs and of the milieu in which they have lodged, as well as other factors such as the patient's immune system. These properties may determine the risk/timing of relapse. Currently, detecting dormant DTCs through tissue biopsy is limited to BM. Furthermore, it is difficult to find biomarkers related to dormant DTCs, or provide prognosis predictions *via* imaging. Fortunately, using innovative approaches, it seems like we are gaining ground. Various technologies that utilized liquid biopsies enable collection of surrogate markers. In addition, it may be possible to predict recurrence based on properties of cells in the primary tumor. Hence, identifying biomarkers in liquid biopsies and in primary tumors that will enable stratification of patients that are at high risk for recurrence, while remaining a great challenge, offers great hope.

Current literature provides evidence for the identification of such markers specifically in breast cancer patients. A recent retrospective cohort study in breast cancer patients with a five-year follow-up after diagnosis found differential expression within the recurring tumors. The differential expression of the proteins was also related to breast cancer subtype. For instance, TNBC patients who recurred had significantly higher expression of Snail protein in their primary tumors compared to those without recurrence, whereas Twist expression was significantly higher in primary tumors of estrogen receptor and progesterone receptor positive breast cancer patients who recurred compared to those without recurrence (154). Notably, both Snail and Twist are transcription factors modulating the epithelial to mesenchymal transition (EMT). Similarly, an increase in mRNA levels of LOXL2, shown previously to induce EMT of dormant DTCs (96, 97) was shown to be associated with a significant decrease in the relapse free survival (RFS) of patients with lymph node-negative breast cancers. Hence, patients with increased levels of LOXL2 mRNA have a higher risk recurrence (96). Whether LOXL2 protein expression in primary breast cancer biopsies can serve as a predictive marker for cancer recurrence and whether it is subtype dependent warrants further investigation. Furthermore, future studies may determine whether combining several EMT markers as predictors of breast cancer recurrence might yield more definitive stratification of patients who are at high risk of recurrence.

Importantly, a 21-gene recurrence-score assay (Oncotype DX, Genomic Health) provides prognostic value. This gene-expression assay is used to assess risk of disease recurrence in hormone receptor-positive, HER2-negative breast cancer patients and to guide decisions regarding adjuvant chemotherapy (155). The assay provides a Recurrence Score (RS), ranging from 0 to 100, indicating low risk (RS < 18), intermediate risk (RS 18–30), or high risk (RS ≥ 31) of disease recurrence. Intermediate risk patients were recently shown by the large prospective TAILORx trial to receive little benefit from chemotherapy in regard to recurrence, with a notable exception for younger patients (156). Hence, early breast cancer patients who are hormone receptor-positive (ER+), HER2-negative with either high or intermediate RS score may benefit in the future from treatments designed to target dormant DTCs.

Indeed, a recent study demonstrated stratification of dormant DTCs in the BM of breast cancer patients based on their NR2F1 expression (157). Importantly, the presence of DTCs in the BM of breast cancer patients was also evaluated previously as potential prognostic marker for breast cancer recurrence (158–160). Bjorn Naume and his colleagues also demonstrated that DTCs status in the BM can identify breast cancer patients who are at high risk of recurrence after receiving adjuvant chemotherapy (161). In addition, a recent study demonstrated that the presence of DTCs in the BM of patients prior to surgery is a significant predictor of late recurrences, particularly for reduced survival in postmenopausal women patients with ER+ disease, lymph node involvement, and large tumors (162). Hence, it warrants further investigation whether DTCs assessment in the BM of breast cancer patients could supplement primary tumor diagnostics such as Oncotype DX and thus may yield more definitive stratification of breast cancer patients who could benefit from preventive treatment.

Importantly, although some gene expression assays of the primary tumor can be used to determine both early and later recurrence and the efficacy of extended adjuvant endocrine therapy in ER+ breast cancer patients, the use of such assays is

not recommended for guiding therapy beyond 5 years (163, 164). Hence, other approaches such as liquid biopsies are emerging as potential prognostic markers to enable stratification of patients who are at high risk for recurrence.

Liquid biopsies, which includes circulating tumor cells (CTCs), circulating cell-free tumor DNA (ctDNA) and extracellular vesicles (EVs), may hold great promise in clinical diagnosis. The presence of CTCs in the peripheral blood of patients after tumor resection denote the existence of minimal residual disease and may provide insight into the process of metastatic spread and enable real-time monitoring of disease progression and therapeutic response [reviewed in (165)]. Several meta-analyses have highlighted the prognostic value of CTCs in various cancers, including breast (166), pancreatic (167), lung (168), colorectal (169) and prostate cancer (170).

A recent study by Sparano and colleagues demonstrated the prognostic value of CTCs in predicting late recurrence of breast cancer patients. The presence of CTCs in peripheral blood samples of hormone receptor–positive breast cancer patients obtained approximately 5 years after diagnosis provided independent prognostic information for late clinical recurrence (171). CTCs positivity was associated with a 13.1-fold higher risk of recurrence. These findings may provide in the near future the premise to stratify patients who may benefit from treatment aimed to target dormant breast DTCs.

The prognostic value of CTCs was also illustrated in prostate cancer patients. A recent study demonstrated CTCs detection in patients who had undetectable PSA levels following radical prostatectomy. Furthermore, these patients with CTCs had an increased risk of biochemical recurrence (defined by an increase of PSA levels) (172). Hence, CTCs may provide future prognostic value to help identify patients after radicalprostatectomy who are at high risk for recurrence. Overall, CTCs are emerging as a potential prognostic marker that may help stratify patients at high risk for cancer recurrence. Whether CTCs are dormant and/or are shed from indolent micrometastases is yet to be explored.

ctDNA is an emerging exciting novel technology in monitoring cancer progression and may guide treatment. ctDNA was shown to be present in plasma samples of many types of tumors that had not apparently metastasized or released CTCs to the circulation (173). Importantly, the total amount of ctDNA at the early stage of cancer patients might be <0.01% of the total circulating cell-free DNA concentration. In a healthy person, the latter is mainly derived from apoptotic leukocytes (165). These extremely low concentrations of ctDNA are approached by several methodologies that rely on a single tumor-specific mutation or a limited panel of mutations known *a priori* to be present in the primary tumor based on previous genomic analysis of the primary tumor. Using this approach Garcia-Murillas and his colleagues performed mutation tracking in plasma DNA of early breast cancer patients receiving neoadjuvant chemotherapy. Detection of ctDNA and mutation tracking of several plasma samples after completion of apparently curative treatment predicted metastatic relapse with a median lead time of 7.9 months over clinical relapse (174). This preliminary study suggests that ctDNA detection may provide prognostic information. Hence, the potential use of CTCs and ctDNA as predictors of late recurrence warrants further investigation. Of note, CTCs and ctDNA are extremely rare. In contrast, extracellular vesicles (EVs) derived from tumor cells are very abundant in blood, are highly stable, and could be used as a source of new biomarkers for personalized diagnosis and prognosis (175). EVs is a global term referring to several different classes of secreted vesicles and includes exosomes, microvesicles, ectosomes, large oncosomes, exosome-like vesicles, and apoptotic vesicles (176). EVs content varies with the type and includes proteins, mRNA, miRNA, long noncoding RNA, circular RNA and DNA, which play a crucial role in regulating tumor growth, metastasis, and angiogenesis. EVs content has been reported to predict recurrence of head and neck and colon cancer after chemotherapy treatment [reviewed in (177)].

Notably, Lev and her colleagues conducted proteomic analysis by reverse phase protein array on EVs content derived from plasma of breast cancer patients. They identified potential markers that can predict the risk of breast cancer recurrence (178). One such marker was HSP70, shown previously to be associated with tumor recurrence (179).

Overall, these exciting emerging technologies such as liquid biopsies hold great promise in developing non-invasive approaches to monitor cancer progression and predict cancer recurrence.

OVERCOMING THE CHALLENGES OF METASTASIS DRUG DEVELOPMENT-DESIGNING TRIALS TO MEASURE CLINICALLY MEANINGFUL EFFECTS DRIVEN BY TARGETING DORMANT DTCs

The end goal for dormant DTC targeting agents is to prevent, delay or minimize recurrence in patients who present with a primary tumor. Another major goal is to prevent, delay or minimize further progression in patients who present with a recurrence. For example, preventing additional recurrences in a patient who has a resectable metastatic lesion at a distant site.

As outlined herein, metastases may arise from dormant DTCs which persist at distant sites, have bidirectional interactions with their microenvironment, avoid the immune system and are undetected by current diagnostic procedures. While the primary unmet need for most cancer patients is preventing metastatic recurrence at secondary sites, the progress made in this area is still limited. Most drug development efforts focus on shrinking existing primary or metastatic tumors in preclinical models, and later in the clinic, and most small molecule and monoclonal antibody drugs are advanced based on their ability to target rapidly dividing cells. The clinical expectation is that overt metastatic tumors will respond and demonstrate a regression of a measurable lesion, with a direct correlation with improved survival and quality of life. Agents that target only dormant DTCs are predicted to have no measurable activity

against proliferating metastatic lesions or primary tumors. Traditional trial metrics of event free survival (EFS), progression free survival (PFS) and overall survival (OS) identify only agents able to control dormant DTCs when this effect also controls other malignant cell growth of a measurable lesion. It is possible that drugs that are effective in current clinical trial designs also have an activity against outbreaking dormant DTCs. Therefore, a new approach that better aligns unmet therapeutic needs with drug development efforts is required.

Practically, upfront trials, where patients who present with a primary tumor are treated with standard of care plus drug/s that can target dormant DTCs are more challenging than trials with patients that already have had metastatic recurrence. This is due to the fact that only a percentage of patient who present with a primary tumor will recur, and due to the fact that the time for a first recurrence can be anywhere from months to many years. As an example, if a trial design is based on EFS at two years, where about 40% of the patients are expected to have an event, the trials must be sufficiently powered to detect improved EFS in the subset of patients who entered the trial. These trials require larger number of patients and they require a longer time of treatment and observation compared to trials in patients who already had relapse. Furthermore, unless agents given to such patients have been specifically shown to eliminate DTCs, they will need to be continuously administered potentially for many years, which is often a challenge, especially when therapies given in conjunction with standard of care that includes chemotherapy. Therefore, it is also less likely that an open label study would be pursued; it is much more likely that a controlled study with a new agent on top of standard of care would be compared to standard of care alone. Implications for time to recruit patients and overall costs is very substantial compared to the option of an open label study. This is especially true given the challenges of identifying the dose that would be optimal for Phase II and III trials. Briefly, since a trial design that utilizes maximum tolerated dose to seek an effect on lesion size is not possible when targeting microscopic dormant DTCs, establishing a selected dose is quite challenging. This is described at more length in the study by Steward et al. (16).

Alternatively, trials in patients that already had a relapse are expected in many cancers to require fewer patients, be shorter in duration of treatment and recruitment time, and importantly be suitable for open label study designs versus controlled studies. As an example, if patients with a certain cancer with well documented historical data have an EFS of 20% at 8 months post-surgical removal (resulting in no measurable disease), a study of a novel agent targeting dormant DTCs with an objective of doubling EFS to 40% at 8 months would require several dozen patients only. An alternate approach could be to target cancers that have a high prevalence of delayed metastasis but that occurs over a manageable time frame. Examples include osteosarcoma, TNBC and melanoma, although the latter also can recur a decade or more later.

Therefore, a development plan in which efficacy of dormant DTCs targeting agents can be determined in smaller trials in patients that have already recurred followed by larger trials in upfront settings is more likely to have support by clinicians, advocates and industry. Drugs that demonstrate efficacy in patients that already recurred can then be tested in the upfront trial scenario, informed by safety, efficacy and PK/PD data from the previous trials.

Novel Trial Endpoints

As noted herein, dormant DTCs are undetectable at distant sites and reside as single/small cellular quiescent cells and or as small indolent micrometastases. Until it becomes possible to directly (e.g., *via* imaging or biopsy), or indirectly (e.g., *via* a biomarker) detect and follow such dormant DTCs and indolent micrometastases, we must rely on traditional clinical end points to assess the activity of drugs that may target and eliminate or control dormant DTCs. Unfortunately, there is no established trial design that completely accomplishes this task.

This is because clinical trials have several flawed assumptions in the context of examining effects on minor populations (180). The first is that clinical trials consider a cancer to be a single entity. Growth of existing lesions or development of new lesions is unequivocally considered progressive disease and thus failure. The second assumption that limits detection of effects on minor populations such as DTCs follows: trial endpoints are indifferent to whether failure (new lesion growth) was due to the outgrowth of dormant DTCs or outgrowth of small tumors below the detection limits of modern imaging. Finally, there is currently no method in clinical trials to distinguish between preventing the outgrowth of small tumor populations associated with dormant DTCs vs eradicating dormant DTCs, since both of these phenotypes would be considered a complete response if accomplished for a long enough duration of follow up.

Herein lies the opportunity for novel trial endpoints to assess agents that affect dormant DTCs and contribute to improved outcomes. Osteosarcoma (OS) provides an excellent disease model for this discussion. OS recurrence is observed in about 40% of patients presenting with localized disease who are treated with standard of care, most commonly to the lungs within a few years of completing therapy (181). About 80% of patients presenting with metastatic disease and who achieve a second complete remission by surgical resection will have further additional recurrences in the lungs. It is unclear if systemic therapy provides benefit when recurrent disease is amenable to resection and thus there is not a standard of care for systemic therapy either before or after surgical resection of lung metastases (181, 182). Thus, the clinical trial community has adopted historically controlled trial designs for this population whereby the null hypothesis is that 20% of patients after resection of the lung lesion will remain without disease at 12 months and an active agent being defined by doubling this to 40% of patients remaining without detectable lung lesions at 12 months (183). (**Figure 3**). Controlled trials in which a therapeutic agent is compared to clinical choice can also be utilized in Phase II or in pivotal or post approval studies with the above time lines informed by historical data. While this trial design captures failure of both growth of subclinical cancer populations and recurrence from DTCs, it is unable to distinguish between these modes of failure.

The hypothesis that we believe needs to be tested clinically involving dormant DTCs posits a limited number of dormant

DTCs and/or dormant indolent tumors either residing alone or in the presence of undetectable micrometastases. Thus, in the presence of undetectable micrometastases even a 100% effective agent at either eliminating dormant DTCs or preventing their outgrowth may fail since the preformed micrometastases may progress clinically. Thus, in order to demonstrate the effectiveness of dormant DTCs targeting therapies in OS, novel trial designs are required. This study design would be based on the hypothesis that some undetectable tumors are too advanced to be affected by drugs that target dormant DTCs. In other words, if microscopic micrometastases resulting from outbreaking dormant DTCs are already present at the time of initial recurrence, they will emerge as additional lesions with or without DTCs treatment. Such a recurrence could be considered an "early failure" and its presence will not truly reflect the efficacy of an agent preventing the outgrowth of dormant DTCs or eliminating dormant DTCs (**Figure 4**). We thus propose an innovative trial design, namely, adding a primary aim of improving disease remission at a later time point (e.g., 2 years following first recurrence in addition to the initial EFS at 12 months post-surgery per point 2 above). This could be an open label study informed by historical data or a controlled study in osteosarcoma. In this scenario, an agent controlling or eliminating dormant DTCs, which would fail by any current trial methodology that removes patients from study at first progression (if any non-dormant cells are present), will have a chance to demonstrate important longer term disease control and clinical benefit missed by current trials.

FUTURE DIRECTIONS

Metastatic recurrence is the major cause of mortality of cancer patients and poses a major unmet clinical therapeutic challenge. Unraveling the mechanisms underlying recurrence which arises years and decades following a protracted period of tumor dormancy may open up novel avenues to prevent disease from recurring. Being able to prevent delayed metastasis, either by specifically targeting dormant DTCs for destruction or maintaining them in the dormant state forever would represent a significant step forward and could save many lives. In this review, we introduced several potential strategies and drugs, some that can be repurposed and may prevent the outbreak of the hibernating DTCs based on growing studies in the field. These strategies aim to maintain the DTCs in indefinite dormancy and/or eradicate them. We also proposed to identify potential gatekeepers in regulation of dormant DTCs and their supportive microenvironment. These strategies could also potentially be complementary to each other. We have also provided various mechanisms and drugs, including some that may be repurposed with a shorter path to treating patients compared to novel compounds in preclinical stages. Notably, each strategy presented here has its complexities and must take into consideration the therapeutic window for treatment and patient stratification that would benefit from such treatment. Ideally, a preventive treatment should be started at the neoadjuvant/adjuvant setting to prevent the disease from ever emerging and/or prevent, delay, or minimize further progression in patients who present with a recurrence.

In order to increase the ability to observe clinical impacts of novel dormant DTC targeting drugs, novel trial endpoints may be required. Since novel dormant DTC targeting drugs are designed to target such DTCs before they exit dormancy, non-dormant cells are likely to progress during treatment and therefore a clinical benefit may only be evident at a later time point (i.e., following the initial "failure" to achieve a clinical outcome). However, later outcomes that would be expected by controlling the dormant DTCs with novel drugs may be expected

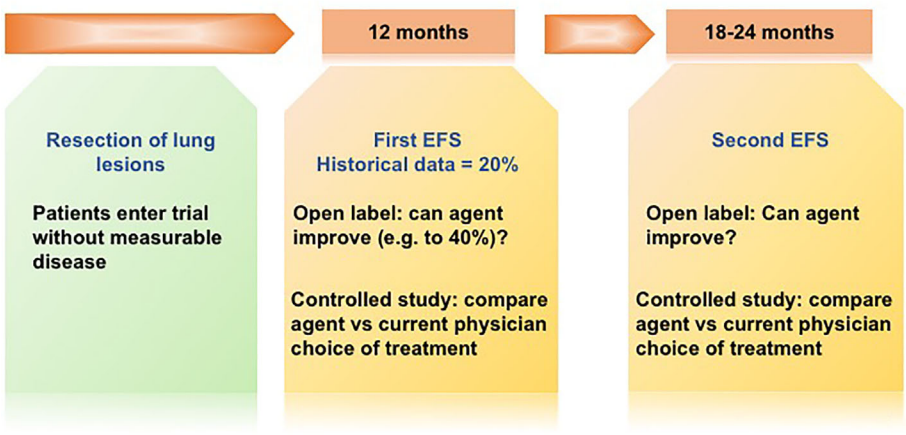

FIGURE 3 | High level clinical trial design. In order to increase the ability to observe clinical impacts of novel dormant DTCs-targeting drugs, novel trial endpoints may be required. We propose an innovative trial design, namely, improving EFS at a later time point, e.g. 6-12 months following the initial EFS at 12 months post-surgery.

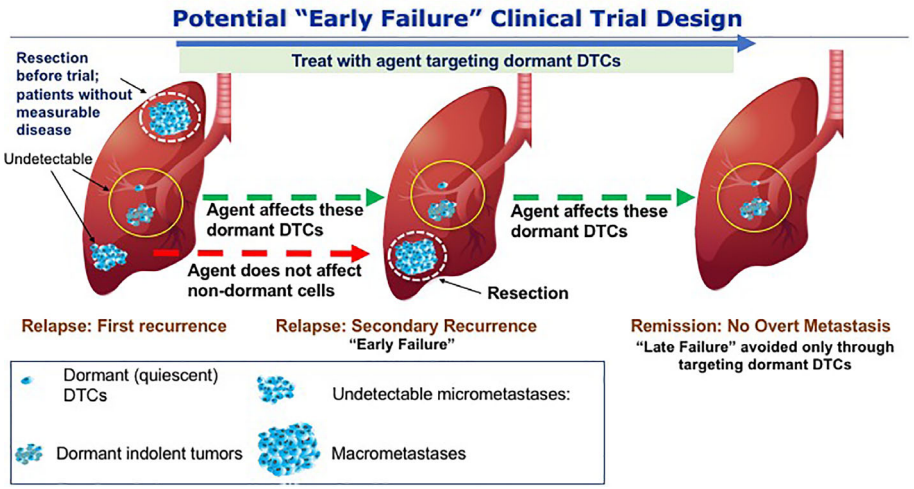

FIGURE 4 | Potential trial to avoid late clinical failure through targeting dormant DTCs. The scenario presented here hypothetically addresses potential drugs that will prevent the outbreak of dormant DTCs in the lungs of OS patients. At the first event of relapse, dormant DTCs that are either quiescent and/or indolent are in the background of proliferating metastases. Upon resection and treatment with the drugs that targets dormant DTCs, cells that are not dormant will not be affected by the treatment and thus may lead to secondary recurrence ("early failure) and therefore a clinical benefit may only be evident at a later time point, i.e., following the initial "failure" to achieve a clinical outcome. By resecting the proliferating metastases and continuing the treatment we may avoid the outbreak of the residing dormant DTCs thus preventing "late failure".

and could have meaningful impacts on disease progression and survival.

Overall, these strategies could potentially open up novel avenues in the battle against cancer recurrence and may develop a strong foundation for developing drugs that would ensure that cancer will never recur.

AUTHOR CONTRIBUTIONS

SS, MI, RH, and DW: research, writing, and editing. DR: conception and writing. DB: conception, research, writing, and editing. All authors contributed to the article and approved the submitted version.

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Early Dissemination of Circulating Tumor Cells: Biological and Clinical Insights

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Circulating tumor cells (CTCs) play a causal role in the development of metastasis, the major cause of cancer-associated mortality worldwide. In the past decade, the development of powerful cellular and molecular technologies has led to a better understanding of the molecular characteristics and timing of dissemination of CTCs during cancer progression. For instance, genotypic and phenotypic characterization of CTCs, at the single cell level, has shown that CTCs are heterogenous, disseminate early and could represent only a minor subpopulation of the primary tumor responsible for disease relapse. While the impact of molecular profiling of CTCs has not yet been translated to the clinic, CTC enumeration has been widely used as a prognostic biomarker to monitor treatment response and to predict disease relapse. However, previous studies have revealed a major challenge: the low abundance of CTCs in the bloodstream of patients with cancer, especially in early stage disease where the identification and characterization of subsequently "lethal" cells has potentially the greatest clinical relevance. The CTC field is rapidly evolving with development of new technologies to improve the sensitivity of CTC detection, enumeration, isolation, and molecular profiling. Here we examine the technical and analytical validity of CTC technologies, we summarize current data on the biology of CTCs that disseminate early and review CTC-based clinical applications.

Keywords: liquid biopsy, CTCs, early dissemination, metastasis, minimal residual disease

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INTRODUCTION

The major cause of cancer related mortality is metastasis (1, 2) which is attributed to dissemination of cancer cells, referred to as circulating tumor cells (CTCs), from the primary site *via* the bloodstream or the lymphatic system to subsequently form secondary tumors in distant sites. The burden of CTCs is strongly associated with cancer prognosis in several cancer types (3). Metastasis was long thought to occur in the later stages of cancer progression in patients with advanced disease. However, a growing body of evidence reports the presence of CTCs at earlier stages of tumor growth, even before the detection of primary tumor (4, 5). CTCs may seed active metastatic tumors or remain in a latent state called tumor cell dormancy that, at some point in time and *via*

mechanisms incompletely defined, exit dormancy to form metastases (6, 7). Comprehensive analysis of CTCs is central to understanding mechanism(s) of cancer metastasis. Molecular profiling of CTCs, and particularly early disseminating CTCs could also lead to discovery of new prognostic and predictive biomarkers to inform patient management. The major challenge for CTC detection and analysis is their rarity in a typically sampled blood volume (10-50ml), and this is observed in most patients with advanced cancer with some notable exceptions such as Small Cell Lung Cancer (8, 9). This challenge of low CTC prevalence is further magnified in early disease settings. In addition to their rarity, CTCs are also genetically and phenotypically heterogeneous (including the spectrum of epithelial to mesenchymal phenotypes). Technologies that can accommodate CTC heterogeneity are critically needed. This mini review will focus on early disseminating tumor cells; in particular, we will summarize methods for identification and characterization of CTCs and give an overview of current knowledge on the biology and clinical relevance of CTC early dissemination.

IDENTIFICATION AND CHARACTERIZATION OF CTCs

CTC enumeration is a well-established biomarker for cancer diagnosis, prognosis, disease progression and prediction of therapeutic response (10). CTCs can be separated and/or enriched from other blood cells by using different technologies that exploit either their physical properties (e.g., size, weight, density, deformability, electrical), or differential expression of molecular markers (commonly intracellular and surface protein expression) or a combination of both (11–15). CTC enrichment techniques employing affinity-based capture methods use antibodies binding to cell surface markers and are based on two strategies; 1) the negative enrichment approach that employs upstream immunomagnetic depletion to remove CD45-positive White Blood Cells (WBCs), though this is rarely achieved to completion, and 2) the positive enrichment approach that captures CTCs and then subsequently removes WBCs (14, 16). While positive enrichment fails to capture cells with low or negative expression of the CTC markers, negative enrichment strategies typically have a lower stringency compared to positive enrichment. The commercial RosetteSep™ CTC Enrichment Cocktail (StemCell Technologies) integrates negative immunoaffinity-based enrichment with density centrifugation. The technology utilizes tetrameric antibody complexes against cell surface antigens found on human hematopoietic cells (CD2, CD16, CD19, CD36, CD38, CD45, and CD66b) and glycophorin A that enables the removal of white and red blood cells from whole blood, thereby enriching for the remaining CTCs (17). Among the positive enrichment approaches, CellSearch® technology is based on immunomagnetic enrichment which uses an epithelial cell adhesion molecule (EpCAM) coated on ferromagnetic particles, with subsequent immunomagnetic separation (18). Alternative CTC isolation techniques are required to capture mesenchymal CTCs or CTCs in the dynamic process of EMT. Additional CTC platforms using enrichment technology include those based on immunomagnetic separation such as a magnetic cell separation system (MACS) (19) and CTC-Chip which still employs an EpCAM-based enrichment approach combined with a microfluidic device (16, 20). The most commonly used antibodies to identify epithelial cells are EpCAM and cytokeratins (CK-19). However, CTCs undergoing epithelial–mesenchymal-transition (EMT) can gradually lose their epithelial characteristics, having no or very low expression of EpCAM and consequentially evade capture and increasingly CTC platforms are combining epithelial markers with others markers, such as mesenchymal markers (e.g., N-cadherin, vimentin or TWIST1), stem cell markers (CD133, CD44, CD34, ALDH1) (21) early apoptosis markers (M30, Bcl-2) or cancer specific markers (e.g., HER2, PSMA) (22, 23). Another platform for CTC detection is AdnaTest (Adnagen AG) that enriches CTCs using a cocktail of antibodies (e.g., EpCAM, MUC-1, AR, Her2) specific to the cancer type (e.g., breast, lung, prostate, ovarian) followed by a subsequent analysis of tumor associated gene expression by RT-qPCR (24). More recently, an approach using an *in vivo* positive enrichment technology named GILUPI CellCollector® (GILUPI Nanomedizin) has been described. This technology allows capture of CTCs directly from the cubital vein of the patient by using antibodies against EpCAM with the advantage of using the total volume of blood and increasing the chance of CTCs isolation (18).

CTCs can be enriched without labelling based on their physical properties. Tumor cells are normally (but not always) larger than most blood cells and this characteristic has been exploited to capture CTCs by size-based filtration using of microfluidic device/cartridges or microchips to separate cells. One option for density-based CTC enrichment is the AccuCyte assay which uses a density-gradient separation technology that integrates a separation tube and a collector device (25). Another example of CTC enrichment technology based on physical properties is the Parsortix™ system that traps cells based on both deformability and size in disposable cassettes with channels that gradually decreases in size to approximately <10μm (26). A combinatory strategy using a microfluidic platforms and a nanotechnology-assisted separation has been also developed for CTC isolation (27). Alternative methods using microfiltration-based enrichment which isolate tumor cells by size include ISET® (Rarecells Diagnostics) (17) and ScreenCell® (28). The electrical properties of CTCs can also be used for tumor-cell isolation by applying a non-uniform electric field through the phenomenon of dielectrophoresis (DEP). Here, a positive (pDEP) or negative (nDEP) force is applied to a cell, moving it towards or away from the electric field source, respectively. Some systems have been described which employ DEP enrichment technologies including ApoStream® (29) and DEParray™ automated system. The latter traps stained cells in DEP cages and is combined with a high-resolution imaging device. Single cells, selected *via* marker expression can be moved within the chip by electrical forces and physically isolated for further genomic analysis (30).

Given the likely loss of CTCs with any enrichment step, CTC platforms have been designed to capture all nucleated cells in the bloodstream. The high-definition single-cell assay (HD-SCA) developed in Peter Kuhn's laboratory (31, 32) and commercially available through Epic Sciences, is based on such a 'no cell left behind' approach, where the entire population of cells in a liquid sample are plated as a biological monolayer onto glass slides and immune-stained for HD-CTC identification. Each slide subsequently undergoes sophisticated image processing to detect rare cells that can be physically picked for genomic analysis or subjected to single cell CYTOF to assess up to 40 proteins per CTC (12, 31, 33). For single CTC image analysis, different tools have been developed for automated processes of classification, sorting, and detection of CTCs for subsequent genetic analysis. A recent study introduced an analysis program called ACCEPT, which classified fluorescent images of single cells from CellSearch® platform as CTCs or not CTCs with an accuracy of 96% (34). In contrast, an automatic tool for label-free CTC detection is also possible whereby CTCs and WBCs are identified directly from bright field microscopy images (35). We cannot describe all the currently available CTC platforms, but refer the reader to a comprehensive recent review (36).

Not all cells detected that are classified phenotypically as CTCs carry genomic aberrations (30, 37, 38) and increasingly phenotypic identification of CTCs is followed by molecular profiling to confirm whether circulating cells, however enriched and isolated, are tumor cells. Molecular profiling of CTCs could also provide unprecedented windows onto the metastatic process, underlying tumor heterogeneity and information on treatment response and resistance (17, 29, 39–44). With the evolving field of single-cell technologies, evaluation of DNA, RNA and protein alterations at the single cell level is now feasible and is being applied to CTCs (**Table 1**) and analysis of paired primary tumor and CTCs has the potential to shed light onto tumor evolution. A study performed on 23 patients showed that shedding of CTCs from the primary tumor is not random; instead, acquisition of copy number aberrations (CNA) is driven by a convergent process across tumor types that ultimately leads to the release of CTCs with complex genomic rearrangements (56). In another study in breast cancer, CTCs resembled CNA of primary tumors and contained alterations associated with brain metastasis with high clonality, suggesting that brain metastasis competent cells had undergone clonal selection (57). Single cell analysis, although exciting can be limited by failures in the technically challenging steps within the workflows. For this reason, expanding CTCs in 2D or 3D cultures or *via in vivo* models could overcome the technical limitations of single CTC analysis and facilitate functional studies. Primary cultures from CTCs have been successfully established in patients with advanced stage cancer (58) which maintained molecular and phenotypic properties of the uncultured primary CTCs, matched genetic alterations of the corresponding primary tumor and could be used to assess molecular changes over time with serial blood draws (59). In contrast, low success of CTC cultures has been reported for patients with early stage cancers, most likely due to the lower abundance of CTCs compared to patients with advanced stage cancer. Optimization of culture conditions and development of eventually CTC cell lines is thus a worthy goal that will improve our understanding of the biological properties of early disseminating tumor cells.

CTCs AS PRECURSORS OF METASTASIS

Metastasis is a complex, multi-step process *via* which cancer cells leave the primary tumor, intravasate and survive in the bloodstream, extravasate, invade and colonize a secondary organ site before growing into a macroscopic metastatic lesion (**Figure 1**) (60). For epithelial tumors, an early step of the metastatic cascade is proposed to occur *via* a dedifferentiation program known as epithelial-to-mesenchymal transition (EMT). During EMT, tumor cells downregulate epithelial markers such as E-cadherin, detach from neighboring cells and acquire a more invasive mesenchymal phenotype (61). EMT program can be stimulated by multiple factors including an activated tumor associated stroma or under hypoxic conditions (62). In addition, the invasive tumor cells upregulate metalloproteinase activity leading to degradation of extracellular matrix and enabling tumor cell migration to reach the vasculature (63). However, recent studies in mouse models have shown that invasion and metastasis can occur independently of EMT (64–66). In particular, E-cadherin may enhance survival during tumor cell detachment, dissemination and metastatic seeding by limiting reactive oxygen-mediated apoptosis (66). These findings may at least in part explain the prognostic role of epithelial CTCs detected by CellSearch® technology in several cancer types (14) and the presence of hybrid phenotypes (epithelial/mesenchymal) in patients with cancer (67, 68).

Aggressive tumor cells can also transition towards a vascular phenotype by expressing endothelial markers and forming blood vessels, a phenomenon called vasculogenic mimicry (VM) (69). Although VM has been described in breast, ovary, lung, prostate, and bladder cancer and has been associated with dissemination and metastasis, it remains a controversial issue, with concerns including a lack of robust discrimination between VM and endothelial blood vessels (70). However, a subpopulation of small cell lung cancer CTCs that co-expressed VE-cadherin (a marker of VM) and epithelial markers had a copy number profile confirming tumor origin, implying that in this aggressive lung cancer, VM may be causally involved in CTC dissemination (71).

CTCs can travel as single cells or as cell aggregates called CTC clusters or circulating tumor microemboli which have been reported for several cancer types including breast, prostate, lung, and colon cancers (72). Although they are detected at a lower frequency and have significantly shorter half-life in the blood than single CTCs (73), CTC clusters are more likely to form metastasis in mouse models (73). CTC clusters can include non-tumor cell types including pericytes, immune cells, platelets and cancer-associated fibroblasts (74) which may support the survival of the clustered CTCs. A recent study identified neutrophils accompanying CTCs in patients with advanced-stage breast cancer where interactions between neutrophils and

TABLE 1 | Summary of studies that performed CTC molecular profiling.

Molecular type	Technology	Readout	Type of Cancer	Main conclusions	References
Genome	Array-CGH/ targeted NGS	CNA/mutations	Colorectal	1) CTCs carry tumour CNA and mutations 2) CTCs represent a small subclone of primary tumour	[Heitzer et al. (45)]
	WES/WGS	CNA/mutations	Lung	1) CTCs carry heterogeneous mutation patterns 2) CNAs are reproducible within CTCS and are selected to lead metastasis	[Ni et al. (46)]
	WES	Mutations	Prostate	1) Feasibility of sequencing whole exome from single CTCs 2) CTCs carry early mutations in tumour evolution	[Lohr et al. (47)]
	WGS	CNA	Lung	1) CNA profiles from single CTCs predict patient's chemosensitivity 2) The CNA classifier correctly assigns 83.3% of the cases as chemorefractory or chemosensitive	[Carter et al. (39)]
	Targeted NGS	Mutations	Breast	1) Mutational heterogeneity in PIK3CA, TP53, ESR1, and KRAS genes between individual CTCs 2) cfDNA profiles provided an accurate reflection of mutations seen in individual CTCs	[Shaw et al. (48)]
	WES	Mutations	Lung	1) CTCs isolated at early stage cancer carry mutation profiles more similar to the metastasis detected 10 months later 2) Potential of using CTCs to predict metastatic genetic lansdscape in early stage lung cancer	[Chemi et al. (30)]
	Targeted NGS	Mutations	Lung	1) CTCs from ALK-rearranged patients resistant to crizotinib are heterogenous 2) Sequencing CTCs at the single-cell level enables to identify resistance mutations	[Pailler et al. (17)]
Transcriptome	RNA *in situ* hybridization	Gene expression	Breast	1) Mesenchymal cells are highly enriched in CTCs 2) Serial CTC monitoring suggests an association of mesenchymal CTCs with disease progression	[Yu et al. (40)]
	RNA-Seq	Gene expression	Prostate	1) CTCs from prostate cancer patients show heterogeneous gene expression patterns 2) Activation of noncanonical Wnt signaling in CTCs from patients progressing under treatment	[Miyamoto et al. (41)]
	RNA-Seq	Gene expression	Breast	1) 17-gene digital signature of CTC–derived transcripts enable high-sensitivity early monitoring of response 2) CTC-RNA signatures may help guide therapeutic choices in localized and advanced breast cancer	[Kwan et al. (49)]
	Padlock probe technology	Gene expression	Prostate/ Pancreas	1) Quantification of AR-V7, AR-FL, PSA, and KRAS mut/wt transcripts in CTCs 2) Padlock probe technology compatible with multiple CTC-isolation devices	[El-Heliebi et al. (50)]
	Whole-genome microarray	Gene expression	Melanoma	1) Melanoma CTCs at advanced disease stages contain heterogeneous cell pools bearing distinct characteristics associated with bone marrow 2) Transcriptional subtyping of melanoma CTCs provides key insights into the molecular mechanisms that regulate metastatic potency	[Vishnoi et al. (51)]
	RNA-Seq	Gene expression	Breast	1) Neutrophils directly interact with CTCs to support cell cycle progression in circulation and to accelerate metastasis seeding 2) CTC–neutrophil clusters may be targeted therapeutically	[Szczerba et al. (44)]
Epigenome	Methylation-specific PCR	Gene specific methylation	Breast	1) Breast cancer metastasis suppressor-1 (RSM1) promoter methylation was detected in a subset of CTCs 2) RSM1 promoter methylation status has biomarker potential in breast cancer	[Chimonidou et al. (52)]
	Multiplex PCR on bisulfite treated DNA	Gene methylation	Breast/ Prostate	1) Hypermethylation at promoters of key EMT genes is not frequent in CTCs 2) Epigenetic heterogeneity among CTCs	[Pixberg et al. (53)]
	Whole genome bisulfite sequencing	Global methylation	Breast	1) Hypomethylation of binding sites for stemness and proliferation associated transcription factors in CTC clusters 2) Na+/K+ ATPase inhibitors enable the dissociation of CTC clusters into single cells	[Gkountela et al. (43)]
	ATAC-Seq	Chromatin accessibility	Breast	1) CTC lines established from breast cancer patients generate metastases in mice with similar pattern as seen in corresponding patients 2) MYC is a crucial regulator for the adaptation of DTCs to the activated brain microenvironment	[Klotz et al. (42)]
Proteome	Antibody barcode microarray	Intracellular proteins	Lung	1)Eight intracellular proteins were measured in more than 80% of CTCs 2) This method is suitable for co-detection of glucose up-take, intracellular proteins, and mutations	[Zhang et al. (54)]
	Single cell western blot	Multiple protein targets	Breast	1)A protein panel comprising of specific targets for breast cancer can distinguish CTCs from WBCs 2) Targeted proteomic methodology is a promising approach for identifying new CTC targets of interest	[Sinkala et al. (55)]

(Continued)

TABLE 1 | Continued

Molecular type	Technology	Readout	Type of Cancer	Main conclusions	References
	Single cell mass cytometry	Multiple protein targets	Prostate	1) Approach that enables multiplex proteomic profiling in addition to morphometric and genomic characterization of CTCs 2) Samples stored for several years can be revisited and analyzed de novo as new protein targets are identified	[Gerdtsson et al. (33)]

This table summarises some of the key studies in the field molecular profiling of CTCs that have shaped our understanding of the potential of CTCs in the clinic as well as for dissecting the biology of cancer. WGS, whole genome sequencing; WES, Whole exome sequencing; CNA, Copy number analysis; NGS, Next-generation sequencing; RNA-Seq, RNA sequencing; PCR, polymerase chain reaction.

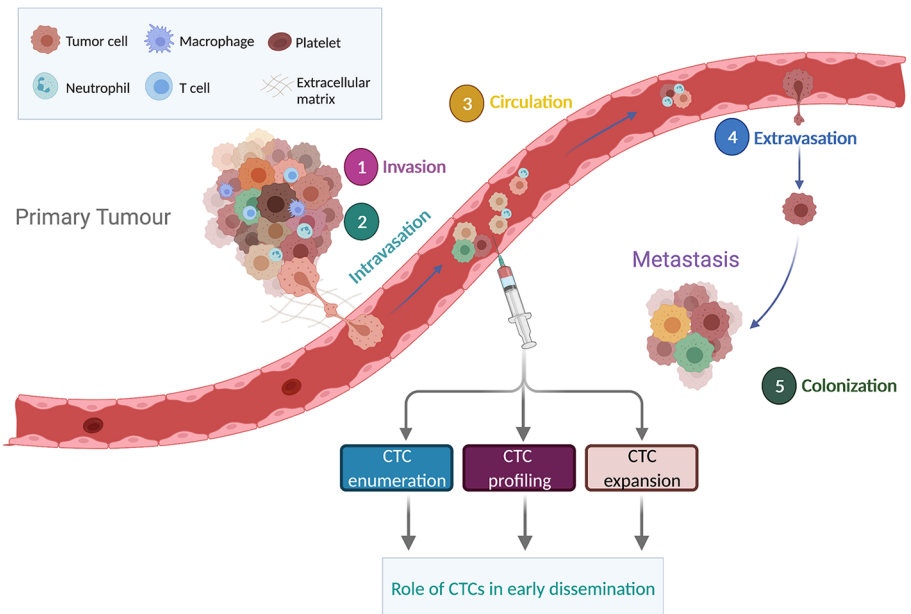

FIGURE 1 | Overview of the metastatic cascade. The metastatic process includes invasion, intravasation, circulation, extravasation and colonization. CTCs detaching from the primary tumor can travel alone or as clusters. Enumeration, molecular profiling and expansion of CTCs in non-metastatic tumors could provide a better understanding on the significance of CTC early dissemination. Figure created in BioRender.com.

CTCs mediated by VCAM1, promoted cell cycle progression and metastatic seeding, opening up new therapeutic vulnerabilities to prevent breast cancer spread (44). Platelets can also interact with CTCs, providing a surrounding 'shield' that prevents recognition by the immune system and protects against shear stress forces within the bloodstream (60). RNA-seq performed on single CTCs revealed that platelet markers were highly expressed in a subset of pancreatic CTCs, supporting the interaction of these two cell types in the circulation (75). CTC survival within the bloodstream may also be achieved by up-regulation of β-globin (a subunit of hemoglobin gene normally expressed by red blood cells), as observed in breast, prostate, and lung cancers, with a consequent reduction of oxidative stress within CTCs (76).

Only a minor fraction of CTCs are thought to complete all the steps of the metastatic cascade (77, 78). CTC extravasation is suggested to occur in a similar manner as leucocyte extravasation, a process involving numerous ligands and receptors expressed by both tumor cells and endothelial cells including selectins, integrins, cadherins, CD44 and immunoglobulin (Ig) superfamily receptors (79). The first steps of distant organ colonization may be partially driven by genetic and epigenetic programs present in a subpopulation of tumor cells at any preceding step of the metastatic cascade before CTCs seed metastasis and molecular profiling of sampled CTCs has the potential to uncover their subsequent competencies and perhaps unveil their tissue tropism. Pertinent to this hypothesis, an *in vivo* genome-wide CRISPR screening performed in breast cancer-derived CTCs identified an upregulation of ribosomal proteins and regulators of the translation machinery in a subset of CTCs that associated with high metastatic burden in mouse models (80). Supporting the notion of predicting tissue tropism, comparison of transcriptomic profiles between breast cancer CTCs associated with brain metastasis and CTCs associated with metastasis to other organs revealed a distinct gene signature associated with brain-homing CTCs (81). In addition, CTCs with alterations in metabolic pathways showed a stronger liver tropism in colorectal cancer (82) and protein ubiquitylation

was identified as an important mechanism of bone marrow metastatic seeding in melanoma (51).

CTCs, DTCs AND TUMOR DORMANCY

Once CTCs have survived within the blood stream and extravasated into a distant site they can reside in a dormant state [often referred to as disseminated tumor cells (DTCs)] for years before 'awakening' to proliferate and cause overt metastasis (83). Several studies have shown that DTCs can be found in the bone marrow of patients without overt metastases, indicating that these cells disseminate early during tumor progression (84). In support of this hypothesis, genetic analysis of bone marrow DTCs from breast, prostate, and oesophageal cancer revealed fewer chromosomal abnormalities in DTCs than in matched primary tumor cells, indicative of a parallel progression model of metastatic growth (85–87). Identification of DTCs, together with an increased understanding and then targeting of the 'awakening' stimuli and mechanism(s) holds potential in improving patients' clinical outcomes although finding, isolating and analyzing DTCs is technically challenging and invasive. Nevertheless knowledge of tumor dormancy and DTCs has improved in recent years. Extrinsic factors including a lack of angiogenesis, immune surveillance, and the balance between proliferation and apoptosis have all been shown to drive tumor dormancy (88). Molecular profiling of DTCs has enabled a better understanding of the cell-intrinsic signals that induce dormancy, such as inhibition of pathways involved in cell-cycle regulation, metabolic signals and autophagy (89). However, only a few studies have investigated whether CTCs from peripheral blood express markers of tumor dormancy (90–92).

In a breast cancer study, CTC subsets were selected for EpCAM negativity, positivity for stem cell markers ($CD44^+$/$CD24^-$) and combinatorial expression of uPAR/intβ1 because downregulation of these two markers has been directly implicated in breast cancer dormancy (90). The $uPAR^+/int\beta1^+$ subgroup of CTCs were found to be more proliferative compared to $uPAR^-/int\beta1^-$ CTCs in *in vitro* assays, suggesting that these two markers could be used to distinguish CTCs that subsequently proliferate *vs* become dormant at distant sites (90). A later study from the same group identified mTOR signaling as a critical determinant in promoting CTC seeding and maintained long-term bone marrow-resident breast cancer cell dormancy (91).

The balance between proliferation and apoptosis has been shown to be associated with tumor dormancy (88, 93). In line with this finding, proliferation and apoptosis markers (Ki67, M30) were measured on CTCs derived from patients with breast cancer who were disease-free for at least 5 years or who relapsed more than 5 years after surgery. The study found that apoptotic CTCs were detected more frequently in patients who remained disease-free compared to those who experienced late relapse, suggesting that the expression of these two markers could be potentially used to predict escape from dormancy (92). In another study, 36% of patients had detectable CTCs 8 to 22 years after mastectomy without evidence of progressive tumor growth. The authors of this study suggest that this could be associated with a failure to complete the final stages of metastasis, which could be potentially being kept in check by a prevailing apoptosis/proliferation balance that maintains a dormant state in distant sites (94). However, the clinical application of CTC detection in the tumor dormancy context still remains unclear. Future research in this field should focus on the identification of CTC molecular features that could distinguish between cancers that are behaving more aggressively from those that will enter a dormant state.

CLINICAL IMPLICATION OF EARLY DISSEMINATION

Analysis of CTCs have enhanced our understanding of cancer biology (61) as well as the potential vulnerabilities of the metastatic cascade. The application of CTC based assays in a clinical setting, especially in early stage disease has been challenging, primarily due to the low frequency of CTCs. Questions often debated in the field of CTC research are whether the low number of single cells analyzed (typically less than 10) are sufficient to capture tumor heterogeneity and if this heterogeneity is better captured in a tumor biopsy (95). However, most tumor biopsy procedures sample a single region or limited number of regions of a tumor and it is often difficult to assess whether the aggressive tumor clones have been captured (96). In comparison, CTCs are cells that have undergone the selection process and have already entered the metastatic cascade, though as mentioned previously, only a minor fraction complete it. Whether these CTCs are indeed a better representation of the aggressive tumor clones than tumor biopsy is yet to be determined, especially in early stage cancers. To this end, in the past decade several studies have explored the potential of CTCs in clinical research and the implications of early dissemination of CTCs in patient diagnosis as well as prognosis. Although technologies for profiling these rare single cells have evolved in the recent years, single cell manipulation and analysis (capture, enumeration, molecular profiling, and bioinformatic workflows) will likely need to be simplified, automated and less expensive to become routinely feasible and taken up in the clinic.

The low prevalence of CTCs in early disease (97–100) clearly hinders extensive studies. In patients with early breast cancer, the TREAT-CTC trial was the first to demonstrate the clinical utility of CTCs using the CellSearch® platform. This trial addressed the requirement of additional treatment to eliminate CTCs post adjuvant chemotherapy and CTC screening was performed at the end of adjuvant chemotherapy in 1317 patients with HER2 negative breast cancer. Of the 95 CTC positive patients, 63 were randomly assigned to observation or trastuzumab administration. The trial demonstrated the feasibility of CTC based screening in an adjuvant setting as well as the higher rate of relapse amongst CTC positive patients (101). More recently, in a cohort of 75 patients' with limited stage SCLC (LS-SCLC, defined as tumor confined within only one lung and/or in the lymph nodes in the mediastinum) the CONVERT trial determined that ≥15 CTCs was as an independent prognostic marker with 60% of patients had detectable CTCs at pre-treatment sampling (102). In localized prostate cancer there was a definite trend towards a positive correlation of CTCs with pathological stage as well as a trend towards prognostic and predictive impact of detecting CTCs with

several studies reporting correlations with patient survival and/or disease recurrence post treatment (103–107). A study in 2014 of patients with chronic obstructive pulmonary disease (COPD), found detectable CTCs in some patients, with these patients developing lung nodules 1–4 years later and with four patients diagnosed with invasive adenocarcinoma and a fifth diagnosed with squamous cell carcinoma, demonstrating the predictive value of CTCs in early NSCLC (4). However, the study also reported false positives in three patients who did not develop overt cancer suggesting the need for further validation using broader CTC detection systems in large nationwide screening programs. Furthermore, in stage I-III NSCLC CTCs collected at surgery prior to tumor resection from the draining pulmonary vein were higher in count compared to sampling of the peripheral blood (1–3,093 *vs.* 0–4 CTCs in the peripheral blood) and although a larger study will be required to validate this finding, CTC count was associated with risk of relapse (30, 108, 109). Strikingly, in a case study within this cohort, genomic comparison of individual pulmonary vein CTCs to the resected primary tumor and a secondary tumor which developed 10 months later, revealed that CTCs had more genomic variants in common with the metastasis than the primary tumor implicating early disseminating CTCs as responsible for disease relapse (29). Further studies using this approach to confirm these findings are warranted.

A further potential utility of CTCs, given the data emerging on early dissemination, is as biomarkers of minimal residual disease (MRD) following treatment with curative intent where tumor phenotype and genotype can be assessed as indicators of (aggressive versus indolent) relapse time-course as a complementary approach to ctDNA monitoring (110). The incomplete primary tumor eradication with consequent persistence of residual cells in the form of CTCs or DTCs remains a major challenge in the clinical management of patients with cancer. The detection of MRD after primary curative treatment has the potential to identify high-risk patients who can benefit from additional treatments and monitoring. The role of CTCs in MRD monitoring has been investigated in several cancer types including breast, colorectal, lung, and prostate cancers (111). In these studies, detection of CTCs at a follow-up time point (ranging from 3 months to 5 years post chemotherapy, accordingly to the tumor types) was significantly associated with unfavorable outcomes. In particular, studies from our group showed that the presence of CTCs (measured by CellSearch®) after one cycle of chemotherapy was associated with worse overall survival in both patients with NSCLC and SCLC (9, 112). Given the proven clinical relevance of CTCs in the MRD setting, ultrasensitive assays are now required in order to detect small number of cells and to capture a broad range of CTC phenotypes (epithelial, mesenchymal or both). More recently, a distinguishing role between CTCs and DTCs has been reported: patients with detectable CTCs in the MRD setting relapsed earlier compared to those with detectable DTCs only, who showed a later relapse (113, 114).

CONCLUSIONS

In the clinic, analysis of CTCs has been used for prognostic stratification of many solid cancers such as breast, small cell lung, non-small cell lung, colorectal, and prostate cancers as well as to monitor disease progression. However, CTCs as a liquid biopsy have not yet fulfilled their undisputable potential to inform of personalized management of patients with cancer which may even extend to the high bar of earlier detection of cancers. The low number of CTCs in the circulation and the sensitivity of the CTC assays currently in use remain a challenge. To this end, intense efforts have been made around the world to standardize CTC based assays to overcome the technical challenges of enrichment, detection, enumeration, isolation, and NGS analyses and to increase assay sensitivity. Developments in the field of CTC enrichment instruments and NGS analyses have elevated CTC studies, bringing exciting insights into biology, heterogeneity and evolution of tumors and begin to illuminate the pathways that underlie tumor dissemination and subsequent steps of the metastatic cascade. These studies include data on CTC heterogeneity, interactions in the blood stream with other cell types, immune evasion, metastatic potential and organ tropism.

However, several unanswered biological questions remain, such as what causes the dissemination of cells into the circulation, what determines the tropism of these CTCs at a metastatic site and further how and which pathways need to be targeted to curb the metastatic potential of these single cells. The answers to these questions could be very different depending on the primary tumor in question and more research must be done to answer them. Furthermore, more studies in large patient cohorts will need to be designed to address the clinical utility of CTCs beyond single CTC and CTC cluster enumeration so that CTC data can be used for individualized tests for drug susceptibility and investigate predictive biomarkers of response to treatments as well as for earlier detection of disease progression. Although, we have come a long way in CTC research the question remains if CTCs are ready for prime time in the clinic. In our opinion, the standardization of CTC assays along with the combining outputs from other liquid biopsy readouts such as cell free DNA, cell free RNA and circulating proteins will help realize their true potential.

AUTHOR CONTRIBUTIONS

FC, SM, and TG drafted the manuscript and AC, DR, and CD evolved the manuscript to the final draft. All authors contributed to the article and approved the submitted version.

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IP-10 (CXCL10) Can Trigger Emergence of Dormant Breast Cancer Cells in a Metastatic Liver Microenvironment

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Metastatic breast cancer remains a largely incurable and fatal disease with liver involvement bearing the worst prognosis. The danger is compounded by a subset of disseminated tumor cells that may lie dormant for years to decades before re-emerging as clinically detectable metastases. Pathophysiological signals can drive these tumor cells to emerge. Prior studies indicated CXCR3 ligands as being the predominant signals synergistically and significantly unregulated during inflammation in the gut-liver axis. Of the CXCR3 ligands, IP-10 (CXCL10) was the most abundant, correlated significantly with shortened survival of human breast cancer patients with metastatic disease and was highest in those with triple negative (TNBC) disease. Using a complex *ex vivo* all-human liver microphysiological (MPS) model of dormant-emergent metastatic progression, CXCR3 ligands were found to be elevated in actively growing populations of metastatic TNBC breast cancer cells whereas they remained similar to the tumor-free hepatic niche in those with dormant breast cancer cells. Subsequent stimulation of dormant breast cancer cells in the *ex vivo* metastatic liver MPS model with IP-10 triggered their emergence in a dose-dependent manner. Emergence was indicated to occur indirectly possibly *via* activation of the resident liver cells in the surrounding metastatic microenvironment, as stimulation of breast cancer cells with exogenous IP-10 did not significantly change their migratory, invasive or proliferative behavior. The findings reveal that IP-10 is capable of triggering the emergence of dormant breast cancer cells within the liver metastatic niche and identifies the IP-10/CXCR3 as a candidate targetable pathway for rational approaches aimed at maintaining dormancy.

Keywords: metastasis, tumor dormancy, tumor emergence, IP-10, CXCL10, breast cancer dormancy, organ-on-a-chip, microphysiological system

INTRODUCTION

Metastatic breast cancer remains a largely incurable and fatal disease. Advances in our abilities to remove and treat primary tumors have not yet translated into sustained success against metastatic disease. Recurrence occurs for ~20-30% of women diagnosed with invasive breast cancer (1). In particular, triple-negative breast cancer (TNBC) is a salient example with 25% of patients succumbing to recurrence within 5 years of their diagnosis (2).

The process of metastasis begins with cells within the primary tumor undergoing a cancer-associated epithelial to mesenchymal transition. This enables motility to disseminate into the circulation followed by extravasation into and colonization of distant organs *via* a partial reversion back to a more epithelial phenotype (3). Outgrowth into overt metastasis then occurs *via* another transition to a more mesenchymal phenotype (4). Strikingly, tumor cells can disseminate even at the earliest stages of primary tumor development (<5 mm) (5–7) and colonize ectopic sites as dormant cells or micro-nodules for years to decades before re-emerging into clinically detectable metastases (8). The signals that drive emergence represent targets for new rationale approaches to prevent metastatic recurrence yet the specific signals and associated mechanisms remain largely unknown.

Herein, we aimed to further our understanding into the developing but still uncertain picture of metastatic recurrence. For our investigations, we focused on the liver metastases as the liver is a major site of metastasis for breast cancer (9, 10) with its clinically evident involvement correlating most poorly with patient survival (11).

It is well-established that inflammatory signals, immune cells, and stromal components are involved in driving the outgrowth of dormant cells (12–18). Regarding the liver, we have shown that non-parenchymal cells of the liver (*e.g.* liver sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells and tissue lymphocytes) can alter cell number and signaling of breast cancer cells, and when activated secrete factors that promote phenotypic changes indicative of emergence (13, 16, 17, 19–21). These studies focused on the role of the local microenvironment, but a hitherto underappreciated area is the involvement of inflammation from distant uninvolved organs.

The gut is the most pertinent distant organ that interacts with the liver as the portal circulation provides most of its blood supply. Dysregulation of the gut can result in increased translocation of bacterial products (*e.g.* lipopolysaccharide (LPS)) and other inflammatory signals. Although the liver routinely handles varying levels of bacterial toxins and inflammatory challenges from the gut, higher loads may overwhelm this homeostatic functioning and lead to overt inflammation, activation of resident liver non-parenchymal cells, and the potential stimulation of dormant tumor cells leading to emergence. Disruption of gut homeostasis has been shown to modulate cancer initiation, progression and dissemination (*e.g.* breast, pancreatic, liver, ovarian, prostate), and drug efficacy (22–24); but the role of gut-derived factors in metastatic disease remains to be determined.

Previously we have established and validated an all-human liver microphysiological system (MPS) – an *ex vivo* 3D organ-on-a-chip microfluidic model of the liver – that recreates metastatic breast cancer that is physiologically reflective of the human situation (19–21). It comprises an all-human biologically replete Liver MPS composed of a human donor-matched hepatic cells (hepatocytes and non-parenchymal cells). Using this *ex vivo* liver metastasis model we showed that quiescent dormant cells in the liver could be stimulated to re-emerge upon exposure to inflammatory products of gut inflammation [*e.g.* LPS/EGF (19)]. This correlated with our 2D data wherein activated non-parenchymal cells produced signals that promote phenotypic changes in breast cancer consistent with emergence (13, 16, 17). Additional insights were provided by a similar but more complex platform that supports interacting human liver and gut modules. The study by Chen et al. (25), found that under pathophysiological systemic inflammatory conditions, a significant non-linear modulation of signaling responses was observed, particularly the CXCR3 ligands (MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11). Together these data compelled us to investigate the possible role of CXCR3 ligands in driving emergence.

Our investigations revealed a significant correlation between the CXCR3 ligand, IP-10, with the TNBC subtype and shortened survival of breast cancer patients with metastatic disease. Using the cellular complex *ex vivo* liver MPS, IP-10 was then found to stimulate the emergence of dormant metastatic breast cancer cells in a dose-dependent manner and that the effect occurs *via* indirect mechanisms.

MATERIALS AND METHODS

Reagents and Cell Sources

Donor matched human hepatocytes and non-parenchymal cells were isolated from excess pathological liver specimens. Patient donors included both males and females with no discernable differences among the genders or donors observed (19). Hepatic niche function and health was unaffected by patient donor background, the presence of breast cancer cells or treatments, and was maintained throughout the experiments (**Supplemental Figure 1**). The cells were obtained from 5 different donors through the Liver Tissue Cell Distribution System, Pittsburgh, Pennsylvania, and funded by NIH Contract # HHSN275201700005C. The liver specimens were provided as separate isolations of hepatocytes and non-parenchymal cells. The latter cells were further purified *via* Percoll gradients as previously reported (26).

The TNBC breast cancer cell line, MDA-MB-231, was purchased from ATCC and transfected with red fluorescent protein (RFP) as described previously (27). The cell line was

Abbreviations: CXCR3, CXC chemokine receptor-3; EGF, epidermal growth factor; HepM, hepatocyte media; IP-10, interferon gamma inducible protein-10; I-TAC, interferon-inducible T-cell alpha chemoattractant; LPS, lipopolysaccharide; MPS, microphysiological system; MIG, monokine induced by gamma; RFP, red fluorescent protein; TNBC, triple negative breast cancer; VEGF, vascular endothelial growth factor.

maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 25 IU/mL penicillin and 25 IU/mL streptomycin (Gibco).

Ex Vivo Metastatic Liver MPS

The *ex vivo* hepatic MPS (Legacy LiverChip® by CNBio Innovations Ltd.) was assembled, seeded and maintained as previously described (19, 26). The functioning and bioengineering behind the MPS (*e.g.* physiological mimicry, fluid flow and oxygenation *etc.*) are also been explained in detail elsewhere (28, 29). Briefly, hepatocytes and non-parenchymal cells were seeded onto scaffolds coated with 1% rat-tail collagen type I (BD Biosciences) at a 1:1 ratio ($6x10^5$ cells/scaffold) in William's E Medium (WE; Life Technologies) supplemented with the Hepatocyte Thawing and Plating Supplement Pack (Life Technologies). Cells were cultured overnight and then the medium was changed to WE supplemented with the Hepatocyte Maintenance Supplement Pack (Life Technologies). After allowing the hepatic tissue to form, MDA-MB-231 cells expressing RFP (500 cells/scaffold) were introduced on day 3. Applicable cultures were treated with 1 μM doxorubicin (APP Pharmaceuticals LLC) on day 7 to 10 and then stimulated on day 13 to 15. The stimulus of 0.5, 1.0 or 5.0 ng/mL IP-10 (PeproTech) was administered in the presence or absence of 50 nM AMG-487 (Tocris). On day 15, scaffolds were removed and fixed in 2% paraformaldehyde at 4°C for 1 hour.

Cell Enumeration and Morphology

Cancer cells within the scaffolds were imaged and cancer burden enumerated as previously described (19). Cell morphology for each cell was calculated based on the width to length ratio and determined by manually tracing and measuring pixel units of the midpoint width and cell length using ImageJ functions determining (16). Imaging instrumentation were provided by and performed at the Center for Biological Imaging, University of Pittsburgh.

Proliferation Assay

MDA-MB-231 cells were seeded at a density of $1x10^4$ cells/well in 24-well plates containing 12 mm coverslips. After 24 hours, cultures were changed to quiescent medium (serum-free RPMI). After an additional 24 hours, cells were treated with 20 ng/mL IP-10, 50 nM AMG-487, 1 μg/mL LPS (Millipore Sigma) + 20 ng/mL EGF (Corning) in serum-free RPMI or HepM plus 10 μM EdU for 4 hours and then fixed with 2% paraformaldehyde at 4°C for 1 hour. Active proliferation was determined using the Click-iT PLUS EdU Alexa Fluor 488 Imaging Kit (Life Technologies) and detected according to the manufacturer's instructions. Coverslips were mounted onto slides and imaged using the Nikon A1 microscope fitted with a 20x objective (Center for Biological Imaging, University of Pittsburgh).

Dormancy Assay

Dormancy was mimicked *in vitro* as reported by Albrengues et al. (30). Briefly, a 96-well plate was coated with 50 μL growth factor reduced Matrigel® (Corning) and incubated at 37°C for 30 minutes. MDA-MB-231 cells ($2x10^3$) were re-suspended in RPMI containing 1% FBS and 2% Matrigel® and seeded into each well. After 24 hours, medium was changed to RPMI containing 0.1% FBS, HepM or either medium plus 4 ng/mL IP-10, 50 nM AMG-487, IP-10/AMG-487, 1 μg/mL LPS + 20 ng/mL EGF or 5 ng/mL TGF-β, then cultured out to and imaged on day 5.

Migration Assay

MDA-MB-231 cells were seeded at a density of $7.5x10^4$ cells/well in 24-well plates. After 24 hours, cultures were changed to quiescent media (0.1% dialyzed FBS; dFBS) overnight. Each well was scratched and then the media was changed to serum-free RPMI, HepM or either medium containing 1, 150 or 500 ng/mL IP-10, 1 μg/mL LPS + 20 ng/mL EGF or 100 ng/mL VEGF. Images were taken at 0, 4, 6 and 8 hours. The scratch width was measured using ImageJ 1.50i software and presented as percent change compared to 0 hours for each scratch.

Invasion Assay

Transwell inserts, 12-well sized with 0.8 μm pores, were coated with 20% growth factor reduced Matrigel® in serum-free RPMI and incubated at 37°C for at least 2 hours in order to solidify. MDA-MB-231 cells were re-suspended at a density of $1x10^5$ cells/mL in either in serum-free RPMI alone, HepM or either medium containing 20 ng/mL IP-10 or 10% FBS. In to each applicable transwell insert, $1x10^4$ cells were added to the apical of transwell inserts and serum-free RPMI, HepM or either medium containing IP-10 or FBS was added to the basolateral compartment. After 48 hours, cultures were fixed with 2% paraformaldehyde in PBS at 4°C for 1 hour. The cells on the upper surface of Matrigel® were removed using a cotton swab. Inserts were then stained with 0.5% crystal violet in dH_2O at room temperature for 10-15 minutes, washed thoroughly with dH_2O and allowed to dry overnight prior to imaging. The color was then extracted from the inserts with a 2% SDS solution and the OD_{550} was determined using a spectrometer.

Breast Cancer Datasets

Expression and survival data on females with Stage IV breast cancer were sourced from the TCGA dataset (n = 19 patients; sourced from the Human Protein Atlas version 19.3, http://www.proteinatlas.org). Patients were identified and selected based on being female and diagnosed with Stage IV breast cancer. Expression data based on breast cancer grade (n = 1832 patients) and hormone receptor status (n = 1903 patients) were obtained from the collated Curtis et al. (31), and Rueda et al. (32), datasets [European Genotype-Phenotype Archive under Accession number EGAS00000000083; sourced from cBioPortal v3.6.11 (33, 34)]. Patients were identified and selected based on their status being known for all estrogen, progesterone and HER-2 receptors. Two patients were removed due to missing estrogen receptor status.

Statistical Analysis

All graphs were generated and statistical analyses were performed using GraphPad Prism version 9 (GraphPad

Software Inc). The Mann-Whitney test was used to assess the *ex vivo* metastatic liver MPS data. Kaplan-Meyer survival curves were evaluated with the Gehan-Breslow-Wilcoxon test. The Mann-Whitney test was used to assess expression differences between TNBC and other breast cancer subtypes. Two-way ANOVA was used to assess migration data, while Student t-tests were used for invasion, proliferation and outgrowth data. Significance was set to p-value < 0.05.

RESULTS

CXCR3 Ligands Are Associated With Tumor Outgrowth

Based upon the significant increase in CXCR3 ligands observed by Chen et al. (25) using a gut-liver MPS, further analysis was performed on previously published data, which modeled dormant-emergent metastatic progression within the Liver MPS (19) (**Supplementary Materials**). Within the prior study three different metastatic states were recreated: i) growing (Hep/NPC plus MDA-MB-231; a mix of actively proliferating and non-proliferating cancer cells), dormant (Hep/NPC plus MDA-MB-231 then doxorubicin; non-proliferating cancer cells) and emergent (Hep/NPC plus MDA-MB-231, then doxorubicin followed by LPS/EGF; reawakened proliferating cancer cells). Analysis of the effluent using multiplex protein immunoassays revealed that CXCR3 ligands were elevated in metastatic niches on day 15 of culture with actively growing populations of MDA-MB-231 cells (growing and emergent), whereas they remained similar to the tumor-free hepatic niche in those with dormant MDA-MB-231 cells (**Figure 1A**). While all three CXCR3 ligands were elevated in niches with actively growing tumor populations, IP-10 (CXCL10; growing 2.2-fold, emergent 1.5-fold) was present at markedly higher absolute levels than that of MIG (CXCL9; growing 1.5-fold, emergent 1.4-fold) and I-TAC (CXCL11; emergent 1.4-fold).

To observe if a connection exists between CXCR3 and its ligands with metastatic disease, their expression levels in patients with breast cancer were assessed (Human Protein Atlas version 19.3, http://www.proteinatlas.org). In those with stage IV disease, IP-10 was not only found to be the predominant ligand present but high expression was also associated with significantly lower survival (**Figures 1B,C**). Furthermore, IP-10 was the most abundant CXCR3 ligand in the *ex vivo* metastatic liver MPS (**Figure 1A**), stage IV patients (**Figure 1B**) as well as the study by Chen et al. (25), Additional analysis of data by Curtis et al. (31), and Rueda et al. (32), found IP-10 to be increased significantly with increasing tumor grade (**Supplementary Figure 2A**). Regarding breast cancer subtypes, it was most abundant in those with TNBC (**Figure 1D**), while lowest in ER+ (estrogen receptor) and ER/PR+ (progesterone receptor) cancer (**Supplemental Figure 2B**). Taken together, these suggest a possible role of IP-10 in metastatic breast cancer progression.

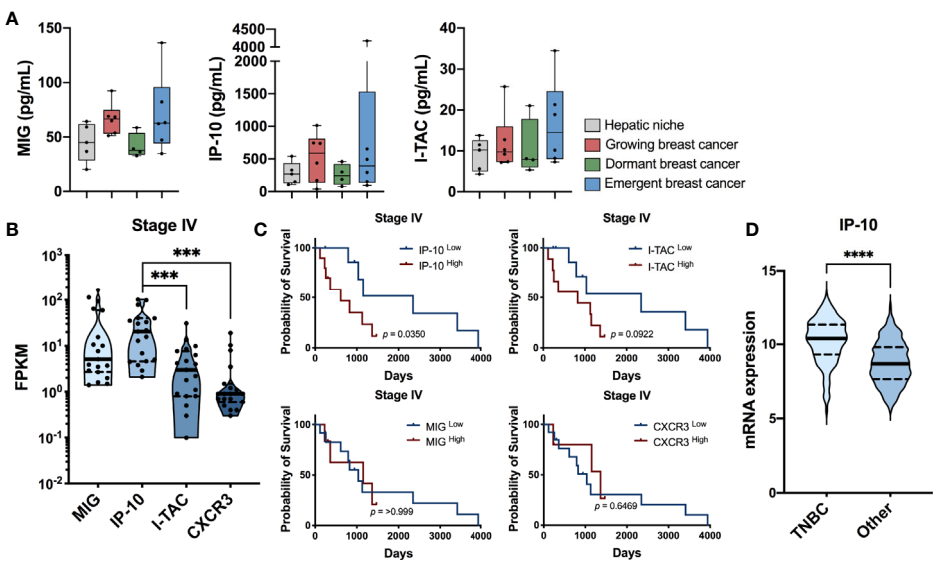

FIGURE 1 | IP-10 is associated with metastatic progression**. (A)** Secreted protein levels of MIG, IP-10 and I-TAC in an *ex vivo* liver MPS for the hepatic niche alone, with growing breast cancer cells, with dormancy breast cancer cells or with emergent breast cancer cells determined using Luminex technology (median with quartiles and range; Mann-Whitney test; n = 4-6 donors). *Ex vivo* data sourced from: Clark et al. (19). **(B)** RNA expression levels human patients with in stage IV metastatic breast cancer (log10 transformation, median with quartiles and range, Wilcoxon matched-pairs signed rank test, *** $p < 0.001$). **(C)** Survival of the aforementioned patients based on high and low expression of IP-10, I-TAC, MIG and CXCR3 (Kaplan-Meyer survival analyzed with Gehan-Breslow-Wilcoxon test). **(B,C)** Human TCGA breast cancer data sourced from: Human Protein Atlas version 19.3 (http://www.proteinatlas.org; n = 19 patients). **(D)** mRNA expression levels of IP-10 in patients with TNBC compared to all other subtypes of breast cancer (median with quartiles and range; Mann-Whitney test, ****$p < 0.0001$). The human breast cancer dataset (31, 32) containing the hormone status data was sourced from cBioPortal v3.6.11 (33, 34) (n = 1903 patients).

IP-10 Promotes Emergence of Dormant Breast Cancer Cells

In order to assess if IP-10 was involved in promoting emergence of dormant cells, our established *ex vivo* metastatic liver MPS method for modeling dormant-emergent progression (19, 26) was employed and modified as per **Figure 2A**. Briefly, MDA-MB-231 cells (expressing RFP) were seeded into the hepatic niche, wherein a subpopulation spontaneously colonize and attain a dormant phenotype (19, 20). To reflect the human situation, the metastatic hepatic niche was then treated with proliferation-targeting chemotherapy (doxorubicin) after which only the dormant MDA-MB-231 cells survive (19, 20). Emergence was then observed by measuring tumor burden after exposing the dormant MDA-MB-231 cells to the stimulus of IP-10 in the presence or absence of an inhibitor of IP-10 (AMG-487). The doses of IP-10 were based around the levels observed in the average levels observed in the effluent of the emergent group presented in **Figure 1A** (1 ng/mL) with additional doses chosen either side of the value (0.5 ng/mL and 5 ng/mL). Exposure of the dormant cells to IP-10 resulted in significant outgrowth of MDA-MB-231 cells and occurred in a dose-dependent manner (**Figures 2B, C**). Further, addition of AMG-487 significantly abrogated the outgrowth effect of IP-10. The MDA-MB-231 cells stimulated with IP-10 also appeared more elongated compared to the control (**Figure 2C**) and was reflected by a reduced midpoint-to-length ratio (**Figure 2D**) suggesting a possible partial reversion to a more mesenchymal phenotype.

Breast Cancer Cell Proliferation Remained Unaltered by Direct Stimulation With IP-10

To determine if the impact of IP-10 on dormant breast cancer cells occurs directly or indirectly, we assessed tumor proliferation following exposure to proportionate levels of IP-10 used in the *ex vivo* metastatic liver MPS. MDA-MB-231 cells were plated at a low cell density to mimic *ex vivo* ratios. The influence of media was also mitigated by performing experiments in both the media for routine tumor cell culturing (RPMI) and in the *ex vivo* metastatic liver MPS (HepM). The proportion of cells actively proliferating was determined by quantifying the number of breast cancer cells that were positive for EdU. IP-10 stimulation was not associated with a significant increase in proliferation in either culture medium (**Figures 3A, B, E, F**). Overall, HepM medium was associated with lower proliferation compared to cells in RPMI. LPS/EGF was associated with increased proliferation compared to control in both mediums, however it was not significant.

Dormancy was then mimicked *in vitro* by embedding MDA-MB-231 cells in Matrigel®. Once again, IP-10 did not significantly impact the outgrowth of MDA-MB-231 cells in RPMI (**Figures 3C, D**). Outgrowth trends were seen for both IP-10 and LPS/EGF

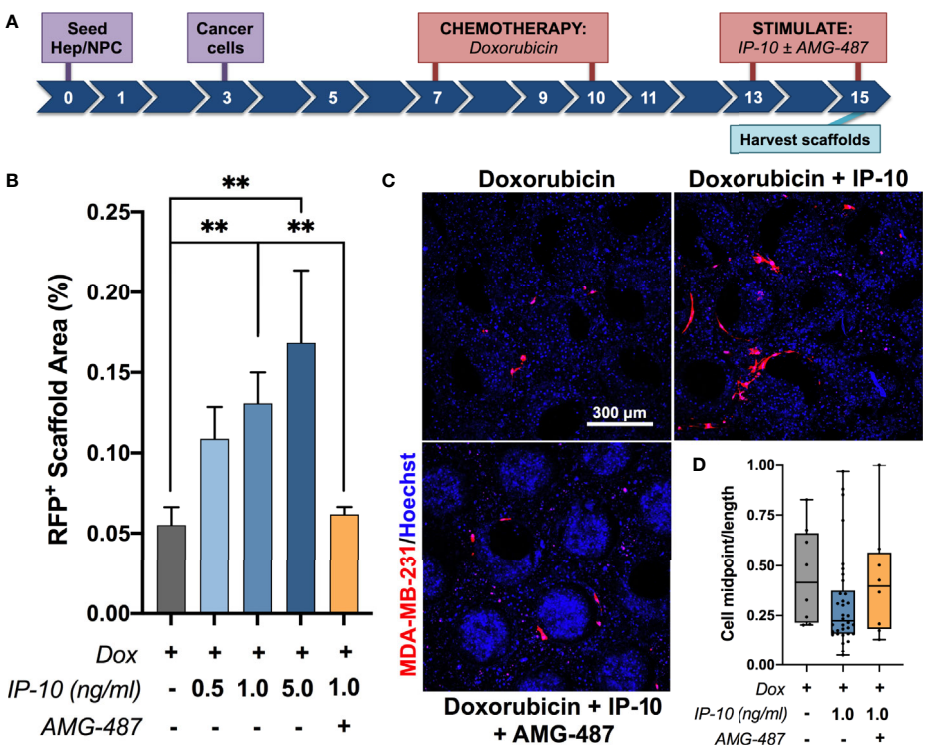

FIGURE 2 | IP-10 promotes the emergence of dormant breast cancer cells in an *ex vivo* hepatic niche. **(A)** Experimental timeline schematic. **(B, C)** Quantification and representative images of the outgrowth of dormant MDA-MB-231 cells following stimulation in the presence or absence of an inhibitor (AMG-487) for 48 hours on day 15 (mean ± SEM; Mann-Whitney test, $^{**}p < 0.01$; n = 2-5 donors). Blue – DAPI; Red – MDA-MB-231 RFP$^+$ cells. **(D)** Quantification of the cell midpoint-to-length ratio of MDA-MB-231 RFP$^+$ cells within the representative images depicted in **(C)** (median with quartiles and range).

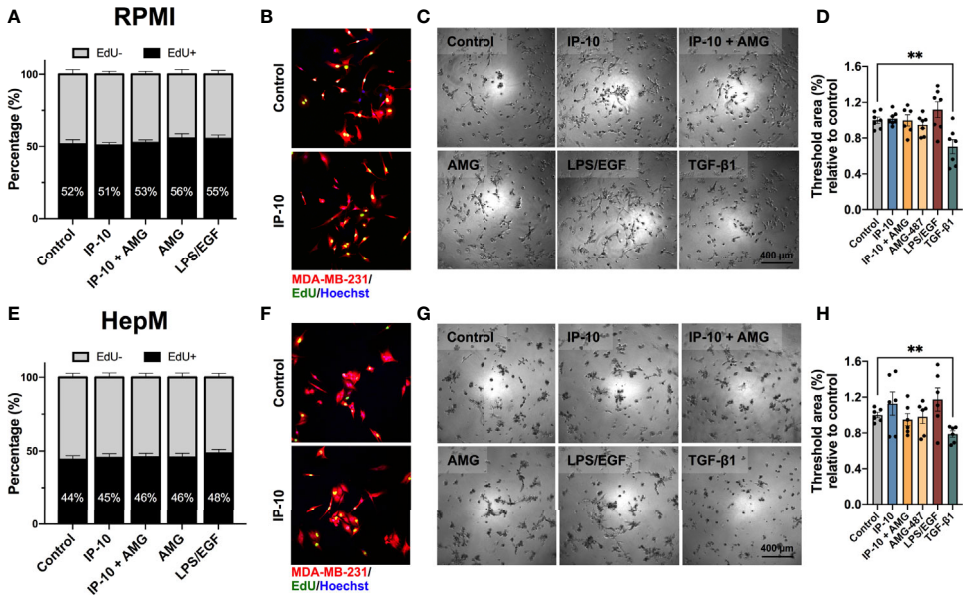

FIGURE 3 | The proliferative activity of breast cancer cells is not directly affected by IP-10. MDA-MB-231 cells cultured in either **(A–D)** RPMI or **(E–H)** HepM medium. **(A, E)** EdU assay for 4 hours to assess proliferation (mean ± SEM; Mann-Whitney test; n = 4). **(B F)** Representative images of EdU assay. **(C, G)** Representative images of a dormancy Matrigel ® outgrowth assay after 5 days in culture. **(D, H)** Quantified outgrowth (mean ± SEM; Student t-test, $^{**}p < 0.01$; n = 3-4).

in HepM media and the latter in RPMI, however, a significant alteration in MDA-MB-231 cell behavior was only observed in the presence of TGF-β1 (negative control) (**Figures 3C, D, G, H**). The data suggests that levels of IP-10 relative to that experienced in the *ex vivo* metastatic liver MPS studies did not affect the proliferation capacity of MDA-MB-231 cells *in vitro*.

Breast Cancer Cell Migration and Invasion Remained Unaltered by Direct Stimulation With IP-10

As MDA-MB-231 cells were observed to transition to a more mesenchymal phenotype in the *ex vivo* metastatic MPS following stimulation with IP-10, the migratory and invasive propensities were investigated. The ability of IP-10 to promote migration was assessed through scratch assays in both RPMI and HepM. A slight increase in the migratory propensity of tumor cells in the presence of IP-10 was observed compared to control in RPMI, though there was no observable difference between the various doses which differed up to 500-fold (**Figures 4A, B**). However, LPS/EGF and VEGF (positive control) were associated with significant migration after 8 hours. Meanwhile, the tumor cells did not migrate in the HepM media even in the presence of the positive control (**Figures 4E, F**).

Invasive capabilities of MDA-MB-231 cells were investigated using transwells coated with Matrigel®. Invasion was promoted by the positive control of 10% FBS, while once again IP-10 only trended with a slight increase (**Figures 4C, D, G, H**). Similarly to the proliferation investigations, IP-10 relative to that experienced in the *ex vivo* metastatic MPS studies did not affect the migratory or invasive properties of MDA-MB-231 cells *in vitro*.

DISCUSSION

Metastasis remains a largely incurable disease and is the major cause of breast cancer-related mortality, with half of the disseminated disease emerging clinically 5 or more years after a seeming cure of the primary tumor (35). Delayed emergence is a result of clinically silent tumor cells which lay as dormant (non-proliferating) cells or nodules in the metastatic site for months to years to decades before suddenly emerging as lethal outgrowths (8, 14, 15). Discerning the operative molecular signals that drive this emergence is key to developing rational approaches to prevent recurrence.

Our model posited that dormant breast cancer cells in the secondary organ (*i.e.* the liver) re-emerge upon exposure to inflammatory cues from a distant, uninvolved organ (*i.e.* the gut) whose homeostasis has been disrupted. Dysregulation of the gut has been linked to the initiation, progression and dissemination of numerous cancers (22–24), however the role of gut-derived factors in metastatic disease remains to be determined. Recent data from an interacting *ex vivo* gut-liver MPS mimicking systemic inflammation identified CXCR3 ligands in particular as being synergistically up-regulated. The receptor itself, CXCR3 is involved both directly and indirectly in tumor progression by regulating tumor proliferation, migration, invasion, chemotaxis and immunity (36). Thus this finding prompted us to investigate if these ligands were involved in regulating metastatic progression of dormant breast cancer cells in the liver.

Our investigations identified a possible role for one CXCR3 ligand in particular, IP-10 (CXCL10), in driving the growth of

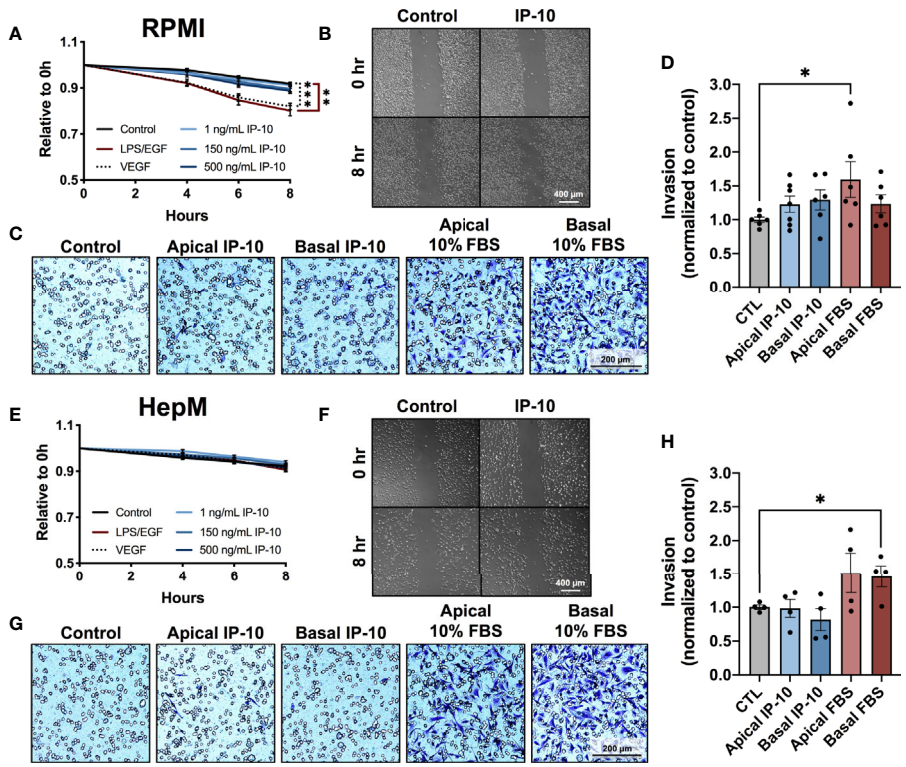

FIGURE 4 | The migratory and invasive properties of breast cancer cells are not directly affected by IP-10. MDA-MB-231 cells cultured in either **(A–D)** RPMI or **(E–G)** HepM medium. **(A, E)** Scratch migration assay measured over 8 hours (mean ± SEM; Two-way ANOVA with Dunnett's multiple comparisons test, $^{**}p < 0.01$, $^{***}p < 0.001$; n = 3). **(B, F)** Representative images of the scratch migration assay at 8 hours. **(C, G)** Representative images of the invasion assay at 48 hours. **(D, H)** Quantification of invading cells (mean ± SEM; Student t-test, $^{*}p < 0.05$; n=2-3).

metastatic breast cancer cells. Analysis of our *ex vivo* metastatic liver MPS – a liver only version of the gut-liver MPS – for CXCR3 ligands revealed increased levels of IP-10 in metastatic niches with actively growing MDA-MB-231 cells more so than MIG (CXCL9) or I-TAC (CXCL11). This *ex vivo* finding was supported by clinical data wherein high expression levels of IP-10 significantly correlated with worse survival in patients with stage IV breast cancer as well as increasing tumor grade. The former is also noted in numerous other cancer types, including melanoma (37), colorectal carcinoma (38), hepatocellular carcinoma (39), prostate cancer (40), and lung adenocarcinoma (41). Furthermore, IP-10 showed highest expression in those with TNBC disease and was the most abundant CXCR3 ligand observed in all three settings (*e.g. ex vivo* inflamed gut-liver MPS, *ex vivo* metastatic liver MPS and stage IV breast cancer patients). As all three ligands bind to and activate the same receptor, CXCR3, we focused on the chemokine with highest levels, IP-10.

With respect to metastatic progression, prior studies have demonstrated that IP-10 can promote dissemination and colonization of numerous cancer types (39, 42–47). However, its effect upon dormant tumor cells post-colonization remains largely unknown. Using our *ex vivo* metastatic liver MPS model, dormant MDA-MB-231 cells exposed to IP-10 were observed to emerge and outgrow. The effect of which was abrogated by AMG-487, CXCR3 antagonist. The importance of IP-10 derived from the metastatic microenvironment is supported by Lee et al. (43), who demonstrated that metastatic melanoma tumor burden was reduced in *IP10-/-* mice compared to wild type on day 14 but not day 7. IP-10 has also been found to be expressed in the normal liver tissue surrounding metastatic colorectal nodules in both mice and patient specimens (38). Our result is consistent with a prior report from Pradelli et al. (48) wherein CXCR3 and its ligands appeared to stimulate the expansion of the osteosarcoma lung metastatic foci in later stages. Furthermore, studies targeting CXCR3 in murine models of breast and melanoma observed inhibitory effects were specifically against tumor metastasis while the primary tumor mass was unaffected (49–51), indicating that CXCR3 has a role in promoting metastasis but not incidence. Combined these imply that host-derived IP-10 plays an important role in promoting the emergence of dormant metastatic cancer cells.

Interestingly, when MDA-MB-231 cells were exposed to IP-10 in the absence of the hepatic niche (*i.e.* alone), only minor changes in their proliferative, invasive and migratory behavior were observed. This was not necessarily unexpected given the intricate relationship that exists between dormant cells and their surrounding metastatic microenvironment. The absence of a

significant effect by exogenously applied IP-10 implies that emergence is likely triggered in the hepatic niche *via* an indirect mechanism. Almost all cells within the metastatic microenvironment, including tumor, immune, stromal and endothelial cells express CXCR3 and are capable of secreting IP-10 (52, 53). This complexity likely accounts for the outgrowth observed in the *ex vivo* metastatic liver MPS, which was not replicated in simpler 2D *in vitro* assays. Within the liver, non-parenchymal cells are both the primary source and most responsive liver cells to inflammatory cues (54). They are capable of altering cell number and signaling of breast cancer cells, and when activated secrete factors that promote phenotypic changes indicative of emergence (13, 16, 17, 19, 21, 28). Subsequently, it is possible that the mechanism by which IP-10 exerts its effect occurs through activation of non-parenchymal cells that then secrete additional factors that stimulate the outgrowth of dormant breast cancer cells.

Determining which cell type within the hepatic niche is triggered by IP-10 to promote the outgrowth of dormant breast cancer cells is an important next step in order to better understand the biology underpinning metastatic emergence and in pursuit of rationale approaches to prevent it. Examination of the literature points towards two specific non-parenchymal cell types – hepatic stellate cells or Kupffer cells/macrophages (43, 55). Hepatic stellate cells express CXCR3 and are responsive to IP-10 in the liver microenvironment (55), while a reciprocal interaction between tumor cells and macrophages at the metastatic site was observed to promote outgrowth (43). Studies elucidating the cell type(s) and signaling network involved are ongoing but lie beyond the scope of the current missive.

In summary, we aimed to identify pathophysiological signals that drive emergence in order to help define candidates whose activities could be targeted to prevent metastatic recurrence. Our studies revealed CXCR3 ligands to be elevated in actively growing populations of metastatic breast cancer cells in a liver microenvironment. In particular, IP-10 was present at much higher levels than MIG and I-TAC, was highest in those with TNBC disease, and its high expression also correlated significantly with shortened survival times in breast cancer patients with metastatic disease. Using an *ex vivo* model of liver metastasis, IP-10 was then found to stimulate the emergence of dormant metastatic breast cancer cells in a dose-dependent manner. However, direct stimulation of breast cancer cells with IP-10 did not significantly change their migratory, invasive or proliferative capacity suggesting that IP-10 acts indirectly *via* surrounding metastatic microenvironment to drive emergence. The findings further confirm that metastatic microenvironment is an integral regulator of dormancy, and highlight the importance of focusing on signals derived by the microenvironment as possible targets for therapeutic strategies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The human cells utilized were determined to be exempt (not human research or human research not engaged) by the University of Pittsburgh IRB and USAMRDC Human Research Protection Office (HRPO).

AUTHOR CONTRIBUTIONS

AC and AW developed the concept, designed experiments, interpreted data, and wrote the manuscript. AC and HH performed the experiments and generated data. LG and DL reviewed the manuscript and contributed to interpretations. AC, AW and DL provided financial support. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.676135/full#supplementary-material

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Conflict of Interest : AW and LG declare a patent on the Legacy LiverChip® now commercialized by CNBio Innovations Ltd. LG also declares consulting fees paid by Zyoxel Ltd. in 2012 but no current relationship.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Therapeutic Targeting of Minimal Residual Disease to Prevent Late Recurrence in Hormone-Receptor Positive Breast Cancer: Challenges and New Approaches

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While the majority of breast cancers are diagnosed at a curable stage, approximately 20% of women will experience recurrence at a distant site during their lifetime. These metastatic recurrences are incurable with current therapeutic approaches. Over the past decade, the biologic mechanisms underlying these recurrences have been elucidated, establishing the existence of minimal residual disease in the form of circulating micrometastases and dormant disease, primarily in the bone marrow. Numerous technologies are now available to detect minimal residual disease (MRD) after breast cancer treatment, but it is yet unknown how to best target and eradicate these cells, and whether clearance of detectable disease prior to the formation of overt metastases can prevent ultimate progression and death. Clinical trials to test this hypothesis are challenging due to the rare nature of MRD in the blood and bone marrow, resulting in the need to screen a large number of survivors to identify those for study. Use of prognostic molecular tools may be able to direct screening to those patients most likely to harbor MRD, but the relationship between these predictors and MRD detection is as yet undefined. Further challenges include the lack of a definitive assay for MRD with established clinical utility, difficulty in selecting potential interventions due to limitations in understanding the biology of MRD, and the emotional impact of detecting MRD in patients who have completed definitive treatment and have no evidence of overt metastatic disease. This review provides a roadmap for tackling these challenges in the design and implementation of interventional clinical trials aimed at eliminating MRD and ultimately preventing metastatic disease to improve survival from this disease, with a specific focus on late recurrences in ER+ breast cancer.

Keywords: tumor dormancy, minimal residual disease (MRD), ctDNA = circulating tumor DNA, molecular residual disease, CTC = circulating tumor cell, breast cancer, adjuvant therapy

INTRODUCTION

In 2019 in the United States, approximately 270,000 new cases of invasive breast cancer were diagnosed and approximately 42,000 women died of the disease. Over 90% of these cases were diagnosed in stages I – III, at a point at which they are potentially curable. Once the disease has left the breast and axillary lymph nodes and become clinically detectable in distant organs, it is no longer curable. Deaths from breast cancer are due to metastatic disease to distant sites that interfere with normal bodily functions. It is estimated that approximately 3.8 million women in the United States have been treated for stage I-III breast cancer. Unfortunately, up to 20% of these patients will experience recurrence at a distant site in their lifetime (1). There is a critical unmet need to identify which women are most likely to recur and to prevent recurrence before it can manifest as incurable overt metastatic disease. The recurrence pattern from natural history studies of hormone-receptor positive (HR+) breast cancer demonstrates that the highest risk of relapse occurs in the first two years of follow-up, followed by a near constant annual relapse rate over the course of a lifetime (ranging up to approximately 2% per year in the highest risk patients), such that approximately 50% of the risk of recurrence for an individual woman is in the period beyond 5 years from diagnosis (2). "Early" recurrence typically refers to those recurrence events that take place within the first 3-5 years, while "late" recurrence typically refers to those events taking place 5 or more years from diagnosis. Adjuvant endocrine therapy, when given for 5 years after initial diagnosis and treatment, has been shown to reduce recurrence risk and improve survival (3, 4). However, this early treatment has less impact on late recurrence risk. The mechanisms driving late recurrences are not clear: both acquired resistance of dormant cells to sustained endocrine therapy or, conversely, the release from dormancy enabled by discontinuation of endocrine therapy have both been implicated (5, 6).

Recurrences that occur beyond 5 years are only modestly reduced by extending the same adjuvant endocrine therapy, i.e., tamoxifen or aromatase inhibitor, for an additional time period (7). Since many women experience late recurrences and extended adjuvant endocrine therapy benefits only a small fraction of women with HR+ breast cancer, dual challenges exist: to find better ways to identify women who are at risk for late recurrence, and develop therapeutic strategies that will further reduce the risk of recurrence in these women. To address these needs, leaders from the National Clinical Trials Network (NCTN) breast cancer committees convened a Clinical Trials Planning Meeting (CTPM) in May 2019, which was followed by an extended planning process to identify new approaches to these issues. This review summarizes the goals and challenges of designing a trial for late recurrence in HR+ breast cancer and recommendations for future trials.

Designing a Trial for Late Recurrence: Challenges and Opportunities

Any trial designed to reduce the risk of late recurrence has several required elements for success, as outlined in **Table 1**. First, it is necessary to identify the population at risk for late recurrence. Several molecular tumor assays have been developed with this goal, and their limited success is described below. All of these are imperfect, as they identify a relatively large population of patients at risk, of whom only a fraction will ultimately relapse. To avoid overtreatment, the ideal approach would be to identify a "real time" biomarker that reflects the presence of residual disease emerging from dormancy where recurrence is imminent but has not yet occurred. Capturing patients at a time when they are in this modifiable window of opportunity allows for intervention to prevent recurrence. Next, a successful trial requires an intervention that is effective against the disease that would otherwise recur, either eliminating these cells or reverting them to a state of permanent dormancy. Such an intervention could be targeted to the cells, the microenvironment in which they emerge, or the immune system, heightening immunosurveillance. Finally, a trial targeting late recurrence must address the complex needs of patients in this setting, who have no signs of overt disease, including the psychological effects of identifying residual disease, the physical and financial toxicity of therapy and the implications of a successful strategy: the need to be monitored and screened over the course of a lifetime. These issues and the various strategies that could be employed are discussed in the sections that follow, with key elements summarized in **Figure 1**.

TUMOR DORMANCY AND MINIMAL RESIDUAL DISEASE DETECTION IN BREAST CANCER

The fact that HR-positive breast cancer can recur many years (or even decades) following diagnosis and treatment for primary disease suggests one of two possibilities: residual tumor cells

TABLE 1 | Design components needed for a late recurrence trial.

Design Component	Description	Examples, Challenges
Method to identify patients at risk	Molecular Tumor Assay	eg. Gene-expression assays (see **Table 2**), other tumor genomic characteristics.
	Minimal Residual Disease Assay	eg. Tumor informed ("bespoke") or agnostic ctDNA assays; circulating tumor cells. *Sensitivity of various approaches are incompletely characterized. Relationship between assay positivity and standard radiographic imaging is unknown.*
Intervention	Pharmaceutical or other intervention demonstrated to reduce both the MRD biomarker and recurrence	eg. endocrine, targeted or immunotherapy. *Tolerability or toxicity may be limiting for many potential interventions*
Endpoints	Clinical endpoint upon which to base success of the intervention	eg. metastasis-free survival. *Most relevant to the goal of the trial but not accepted FDA endpoint for drug registration. Long follow-up may be required.*
Patient Reported Outcomes (PRO)	Instruments that assess impact of identifying MRD on quality of life	eg. Global QOL, CTCAE-PRO.

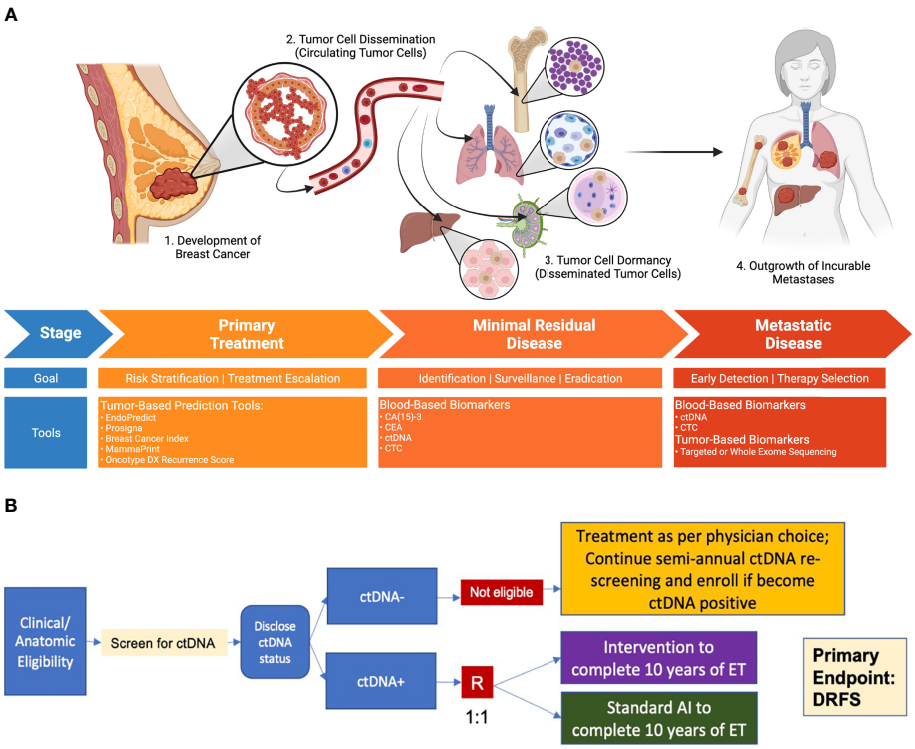

FIGURE 1 | Process by which primary tumors progress through dormancy to distant metastatic disease, and opportunities for intervention. **(A)** Breast Cancer Treatment Continuum: Minimal Residual Disease as a Therapeutic Opportunity to Prevent Late Recurrence. New approaches to identify at-risk individuals and evaluate therapies to reduce late recurrence may focus on the period following standard upfront treatment. The detection of minimal residual disease through blood-based surveillance tools might enable the identification of individuals at highest risk of metastatic recurrence, for whom escalated therapies may have the greatest potential benefit. The principal goal of such interventions is the prevention of metastatic recurrence. **(B)** Example Design Schema for a Phase 3 ctDNA-Guided Late Recurrence Trial. The use of highly sensitive ctDNA detection methods in patients at high clinical risk permits the identification of those most likely to recur, for whom investigational therapies could be evaluated (bottom). Those without detectable ctDNA may continue regular ctDNA surveillance, becoming eligible for therapeutic intervention if ctDNA is subsequently detected. Clinical endpoints of particular importance include distant recurrence-free survival, and overall survival. "R" denotes randomization step; DRFS, distant recurrence free survival; ET, endocrine therapy.

disseminated in the body proliferate at a very slow rate, until these growing tumors become clinically evident; or residual tumor cells lie dormant for prolonged periods, until they escape and grow more quickly to form detectable tumors. Conclusive evidence to support either the former (indolency) or latter (dormancy) model is lacking, and it is likely each phenomenon contributes to some cases of metastatic recurrence. However, based on many factors, including observed rates of tumor growth in primary disease and their recurrences, temporal and spatial patterns of recurrence, and biologic evidence to support the existence of dormant states (reviewed in other accompanying manuscripts), dormancy is favoured as a key contributor to late recurrences of HR+ disease (8, 9).

In either case, dissemination of microscopic disease that is not removed by locoregional treatment (surgery and radiation) is a necessary prerequisite. Circulating tumor cells (CTCs) in the blood are implicated in early dissemination (10). The existence of, and ability to detect disseminated tumor cells (DTCs) in the bone marrow of patients diagnosed with breast cancer has been well-recognized and studied for several decades. DTC detection frequencies of ~25-30% at the time of early breast cancer diagnosis have been observed in large, pooled analyses, where DTC detection is associated with high-risk tumor features, such as tumor size, nodal positivity, and tumor grade (11). In multivariable analyses, the presence of DTCs is independently associated with the risk of recurrence (though limited data are available to specifically assess late recurrence of HR+ disease) (12, 13). Bone marrow DTCs in patients without clinically evident metastases are one example of "minimal residual disease" (broadly defined as persistent evidence of cancer that is not detectable with standard clinical or radiographic assessments, MRD), which may provide the seeds for subsequent recurrence. However, their direct role in the metastatic cascade is uncertain, and not all patients with bone marrow DTCs at diagnosis experience recurrence. Numerous potential explanations for this exist, including the elimination of residual disease with adjuvant therapy, long term control or elimination by the host immune system, or a lack of the necessary cell-intrinsic or extrinsic (eg. microenvironmental)

conditions to permit tumor outgrowth, resulting in either elimination of disseminated cells or their persistent dormancy.

An ability to identify and assess MRD over time would permit both a better understanding of its natural history, and perhaps better enable accurate individualized risk assessment at time points remote from diagnosis that could guide clinical interventions. Unfortunately, the bone marrow biopsies necessary to assess DTCs are invasive and uncomfortable, limiting their clinical application. However, the rapid development of several technologies for liquid biopsy from circulating blood offer some novel approaches to this problem. Liquid biopsy to detect MRD has focussed on two main approaches: (1) the detection of intact tumor cells in the circulation (circulating tumor cells, CTCs), and (2) the detection of DNA released from tumor cells detected in the circulation as cell-free DNA (circulating tumor DNA, ctDNA) (14). The technologies underlying each of these approaches (recently reviewed elsewhere (14, 15)) depend on the identification of tumor specific features not normally present in blood samples. For CTCs, this includes immunophenotypic characteristics of circulating cells such as EpCAM (Epithelial Cell Adhesion Molecule) and cytokeratin (together with absence of leukocyte markers) that mark their tumor origin (10), whereas for ctDNA, the presence of somatic alterations (point mutations, copy number alterations) or methylation patterns can distinguish ctDNA from other cell free DNA (cfDNA) released by normal cells.

Several recent reports have identified strong associations between ctDNA or CTC detection and subsequent breast cancer recurrence, as described below (16–20). In general, detection of MRD using current liquid biopsy techniques has yielded lead times (ie. the time between sampling and clinical presentation with metastatic disease) of about 1-2 years. While it is possible, or perhaps likely, that these lead times may increase as assay detection sensitivities increase, several important gaps exist in our current understanding of MRD detection that would impact its clinical utility. Chief among these is the proportion of detectable cases that represent true MRD, which could not be simultaneously detected using other standard imaging techniques, as opposed to radiographically overt but clinically occult metastatic disease. This distinction is crucial, since true MRD may present an opportunity for cure, whereas established breast cancer metastases are understood to be generally incurable with currently available therapies. Prospective studies that incorporate serial imaging with concurrent sampling for liquid biopsy will be required to address this question. A second important and unanswered question is whether detectable MRD represents disease that remains dormant but at risk for later escape, or represents instead a later stage of cancer outgrowth following escape from dormancy. This distinction is important, as relevant therapeutic strategies may differ in each scenario (discussed further below). Additional clinical evaluation of evolving liquid biopsy technologies should provide some insight, as will monitoring of dynamic changes in MRD characteristics in response to proposed therapeutic interventions. However, it remains unclear whether changing the therapeutic approach at the time of MRD detection or whether changes in MRD after modifying treatment will correlate with improved clinical outcomes.

Despite the existing uncertainties noted above, several studies have been recently been launched to investigate the use of ctDNA MRD as a selection marker for interventional trials. These include studies of HR+ and HR- breast cancer, which principally focus on the early recurrence period. c-TRAK-TN (NCT03145961) evaluated immunotherapy in patients with triple negative breast cancer, DARE (NCT04567420) and LEADER (NCT03285412) are evaluating CDK4/6-inhibitors in ER+ disease, and ZEST (NCT04915755) is evaluating a PARP inhibitor in BRCA-related or triple negative breast cancer.

The elimination of MRD to prevent breast cancer late recurrence and achieve clinical cures will require therapeutic intervention with systemic treatments that will bring costs – both financial and in the form of treatment toxicity. Ensuring that costs are accompanied by the greatest likelihood of benefit could be achieved by the identification of individuals most likely to recur. While in other adjuvant settings this is generally achieved by using population-based tools for risk stratification, it is possible that MRD detection could ultimately binarize individual risk (ie. if patients with MRD at late timepoints invariably experience recurrence). However, the current costs and practical logistics of liquid biopsy approaches (combined with the overall low risk in unselected women with HR+ breast cancer) will nonetheless require tailoring any MRD-based intervention strategy to populations with some meaningful risk threshold. The application of various existing risk-stratification tools based on standard clinicopathologic variables could permit the development of such a strategy. In the remainder of this review, we discuss the key considerations for the development and evaluation of a therapeutic strategy to prevent late recurrences of HR+ breast cancer.

IDENTIFYING THE POPULATION AT RISK

Tumor-Based Features

The overarching goal of risk stratification is to identify a population at high enough risk for metastatic recurrence in whom escalating treatment may be warranted. While late risk of recurrence can continue beyond 10 years, many population-based tools have only examined the 5-10 year window (1, 2). Risk thresholds could be set at varying levels to enrich a study population, based on the intensity of the intervention of interest, with 10% to 15% risk of distant recurrence from years to 5 to 10 resulting in feasible trial size (e.g. up to several thousand patients) with the potential for meaningful results in a definitive Phase III adjuvant trial. While higher risk level thresholds could limit feasibility and the rate of accrual since these patients are fewer in number, inclusion of patients with higher risk would enrich for events, enabling a larger absolute magnitude of benefit, and thus a higher benefit/risk ratio for a given intervention.

Both anatomic and biologic tools based on the excised primary tumor exist to assess recurrence risk. Standard clinicopathologic features, including anatomic stage (tumor

size and lymph node involvement) and grade of the original tumor continue to provide information on risk of recurrence for at least 20 years from diagnosis, with risk of distant recurrence beyond year 5 ranging from 10% to 41% in the Early Breast Cancer Trialist Collaborative Group analysis of outcomes for almost 63,000 trial participants (1). Many patients in this initial work had been treated on older studies, and an updated analysis of 86,000 participants on 110 trials found that with contemporary therapy (patients diagnosed after 2000) the risk of recurrence beyond year 5 is approximately 25%. A tool based on standard clinicopathologic features, the Clinical Treatment Score Post 5 Years (CTS5) has recently been developed and described (21). This web-based, widely available calculator uses initial tumor size, grade, patient age and nodal status to classify patients as low (<5%), intermediate (5-10%) or high risk (>10%) for distant recurrence during years 6-10 following diagnosis. While simple, based on readily available information, and validated in post-menopausal women, this tool is based on limited data for extremes of tumor size or nodal status, and diminished validity has been observed among premenopausal women (22).

Gene expression classifiers have provided important molecular insights into breast cancer recurrence, and the expression of many genes – including those involved in proliferation and estrogen signaling – is correlated with risk of recurrence. The goal of genomic assays is to reliably define the risk of recurrence so that patients are appropriately treated with adjuvant systemic therapy. Several commercial assays have been developed and validated as prognostic tools and are used routinely to stratify patients for the delivery of adjuvant therapy (**Table 2**). These assays, which are performed on primary tumor tissue, include the immunohistochemical 4 (IHC4) protein test, 21-gene Recurrence Score (OncotypeDx), PAM50 intrinsic subtype (ProSigna ROR; risk of recurrence), 12-gene EndoPredict Score (EPClin), 70-gene signature (MammaPrint), and 2-component Breast Cancer Index (BCI; HOXB13:IL17BR). These genomic assays offer prognostic information on the anticipated natural history and risk of recurrence, having been analyzed in prospective-retrospective studies and, depending upon the test, prospectively validated in large, randomized trials, such as TAILORx (OncotypeDX) (34) and MINDACT (MammaPrint) (32), and RxPONDER (OncotypeDX) (35). In addition, genomic assays have the potential for predictive utility to help guide individual treatment decisions, such as omission of adjuvant chemotherapy. For instance, in the overall population with an intermediate recurrence score in TAILORx, defined as RS between 11 and 25, patients with node negative disease did not experience a significant clinical benefit with the addition of chemotherapy to endocrine therapy at 9 years (34). RxPONDER extended this finding to post-menopausal women with 1-3 involved lymph nodes, in that there was no subgroup of post-menopausal women with a recurrence score of < 26 who benefited from the addition of chemotherapy at 5 years (33). Similarly, in the MINDACT study, the MammaPrint assay was able to identify patients with high clinical risk but low genomic risk who had a relatively favorable prognosis in the absence of systemic chemotherapy (32).

While these assays each have demonstrated prognostic utility within the first five years from diagnosis, one study performed a direct comparison of various assays to evaluate their relative prognostic capabilities from years 0-10. In the TransATAC trial, tumors from 774 of the postmenopausal women with HR +/HER2- breast cancer were characterized with the following tests: OncotypeDx, BCI, Prosigna ROR, EPClin, IHC4, and CTS5 (36). For late (between 5-10 years) distant recurrence, BCI, ROR, and EPClin provided independent prognostic information for women with node-negative disease, as well as in a small population of patients with node-positive breast cancer. These three assays can also identify a subset of patients with anatomically low risk tumors who are at higher risk of recurrence, or who have anatomically higher risk tumors that have genomically lower risk disease. An analysis of EP using separate clinical trial cohorts (ABCSG-6/8) found that 22% of

TABLE 2 | Genomic risk assessment tools.

Tool	Description	Level of Evidence
COMBINED GENOMIC/CLINICAL		
EndoPredict (23–25),	RNA based, 12-gene assay combined with tumor size and nodal status, developed in pre- and post-menopausal women treated with tamoxifen	- Validated ~2600 post-menopausal women in ABCSG6/8 and TransATAC
Prosigna ROR (26–28),	PAM50-based 46 gene-signature developed in pre- and post-menopausal women treated withoutany adjuvant systemic therapy. Includes tumor size	- Validated in ~2100 women in ABCSG 8 and TransATAC and in ~2500 Danish women cohort for 10-year risk of recurrence
GENOMIC		
Breast Cancer Index (29, 30),	Combines the 2-gene HOXB13:IL17BR ratio with the molecular grade index from five proliferation genes in a linear model; developed in post-menopausal patients with HR-positive, node negative breast cancer. The node positive assay includes tumor size	- Developed on blinded retrospective analysis of 588 Swedish women treated on tamoxifen trial
MammaPrint (31, 32),	70-gene RNA expression profile	- Level 1 evidence for addition of systemic chemotherapy to adjuvant anti-estrogen therapy
Recurrence Score (OncotypeDx) (33)	21-gene signature developed in HR-positive, N0 patients. RxPONDER demonstrated discrimination extends to post-menopausal women with disease involvement in 1-3 nodes	- Designed to predict benefit of addition of systemic chemotherapy to adjuvant anti-estrogen therapy; Level 1 evidence for this

patients with node negative disease had tumors that were EPClin score high (with a predicted 10-year distant recurrence-free rate of 87.0% (95% CI 82.6%-91.7%) and that 30% of patients with node positive breast cancer had tumors which were EPClin score low (10-year distant recurrence-free rate of 95.6% (95% CI 92.2%-99.1%) (23). Similarly, BCI has reported on two cohorts of patients with low anatomical risk (T1N0) breast cancer in which 32% and 36% of tumors were classified as BCI high risk associated with reduced distant recurrence-free survival 86.7% and 89.6% at years 5-15 and 5-10 compared with 95.4% and 98.4% for patients with BCI low risk tumors in each cohort respectively (37). In 402 patients with node-positive (N1) breast cancer, ~20% were classified as BCI low risk, with a 15-year distant recurrence risk of 1.3% (30, 38–40).

Taken together, the available data support the use of both anatomic and tumor-based genomic classifiers to identify patients at increased risk of late recurrence for inclusion in a study of late recurrence interventions. Targeting a 10 to 15% risk of recurrence over years 5 to 10, such an approach would include anatomically high risk (e.g. Stage III disease) and reserve eligibility based on genomic high risk to those who are anatomically at lower risk. Ideally, all enrolled patients would have tumor-based molecular testing for subsequent correlation of genomic and anatomic risk, including in the higher stage cohorts for which limited information is currently available. Some important unknowns remain in the application of these tools to define a trial population, including the relationship between genomic classifiers and the prevalence of detectable MRD, as well as the impact of emerging therapies which may be incorporated into early adjuvant therapy of high-risk disease (e.g. cyclin-dependent kinase 4/6 inhibitors (41)) on their prognostic estimates. Furthermore, limitations of the existing risk classifiers include a paucity of validation in pre-menopausal women for several of the approaches, and uncertainty in whether any assays may be predictive of therapeutic benefit for various treatments that may be considered.

Blood-Based Biomarkers

There is significant interest in the clinical development of minimally invasive tests, such as a blood-based biomarkers, that could help determine if an individual patient is or remains at high risk of recurrence. Ideally, a reliable marker would detect the presence (or likelihood) of minimal residual disease (MRD, as described above) to identify individuals at high risk of recurrence prior to radiographically detectable incurable metastatic disease (e.g., during a period of tumor dormancy). These patients may benefit from modification or escalation of therapeutic strategies to ultimately decrease the likelihood of developing metastatic breast cancer. This is an attractive approach, as it may detect the development of resistant disease in real-time, as opposed to basing prognostic risk upon clinical, pathologic or genomic features of the historical primary tumor.

While serial assessment of tumor markers, such as cancer antigen (CA)15-3 (the soluble moiety of the MUC-1 glycoprotein) and carcinoembryonic antigen (CEA) are used sometimes in patients with metastatic breast cancer, serum tumor markers are not recommended in the surveillance of patients treated for early breast cancer due to (i) concerns with sensitivity, (ii) the finding that the positive predictive values of these markers decrease over time, and (iii) lack of evidence that serum marker measurement improves clinical outcomes (42, 43). A renewed interest in the assessment of circulating markers has emerged with the development of newer tests for MRD, including CTCs and ctDNA, and emerging clinical results demonstrating their prognostic effects, including in the setting of late recurrence.

In the Eastern Cooperative Oncology Group E5103 trial, the presence of CTCs (i.e., at least 1 CTC per 7.5 cc whole blood) detected 4.5-7.5 years following diagnosis, as measured using the EpCAM-based CellSearch platform, was associated with an adjusted relative risk for distant recurrence of 13.1 [95% CI: 4.7 to 36.3] after a median follow-up of 2.6 years (41). Of note, only 5% of patients in E5103 were identified as having CTCs present. The median time to recurrence was 2.8 years (range, 0.1-2.8 years) among the CTC-positive patients. In a second study. (SUCCESS A), the presence of CTCs 5 years after chemotherapy in patients with HR+ breast cancer was also shown to be an independent predictor of recurrence-free survival in multivariable analysis (HR 5.95, 95% CI: 1.14 – 31.16, p = 0.035) (17). Studies with other platforms, including non-EpCAM based technologies, are ongoing to further evaluate the potential prognostic and predictive effects of CTCs. Recognizing that the studies described did not incorporate serial radiographic imaging (which is not routinely performed in this setting), the degree to which CTC detection in these studies represented true MRD is unknown.

In addition, a number of recent prospective-retrospective studies have described the evaluation of minimal residual disease (or in this case, "molecular residual disease") with ctDNA in patients with early-stage breast cancer. Some platforms have used bespoke assays which target somatic alterations identified in the primary tumor (18) while others are agnostic to alterations identified in the primary (19). In a cohort of 144 patients and at a median follow-up of 36.3 months, molecular residual disease was detected in 29 patients, which was highly prognostic in a time-dependent model (HR, 32.8; 95% CI, 13.5-79.2; $P < .001$). The median lead time between ctDNA detection and relapse was 10.7 months (95% CI, 8.1-19.1 months) (18). While this analysis included all breast cancer subtypes, 51 patients had HR+/HER2- breast cancer, and the hazard ratio was not definable because no patients experienced relapse in the ctDNA-negative group, with a median lead time of 13.3 months for those with ctDNA-positivity (95% CI, 2.1 months to undefined; $P < .001$). In addition to identifying MRD and its associated risk of recurrence, ctDNA analysis can also identify genomic alterations, such as ESR1 mutations (which confer ligand-independent activation of the estrogen receptor), which may help define mechanisms of resistance to standard therapy (eg. aromatase inhibitors) and inform therapeutic interventions, as well as provide opportunities to assess response to these treatments, such as by dynamic changes in variant allele frequency (16).

INCORPORATION OF MARKERS INTO A PROSPECTIVE THERAPEUTIC TRIAL

While CTCs and ctDNA have the potential to further discriminate risk, predict potential benefit to therapy or provide surrogate markers of response, these blood markers are not yet ready for inclusion in a definitive clinical trial in the late adjuvant setting. An integral marker for a large adjuvant study must satisfy a number of criteria, which - in the case of CTCs and ctDNA for late recurrence - remain to be further defined. Chief among these, from a screening perspective, include the relationship between minimal residual disease positivity *via* CTCs or ctDNA and scan-detectable, subclinical metastatic disease, and the prevalence of CTC or ctDNA positivity in a population eligible for a large adjuvant study. It also remains unclear whether the identification of these markers represents a tumor that has already escaped from dormancy or is at imminent risk of doing so. Further investigation will also clarify if the detection of CTCs and ctDNA capture similar or distinct groups of patients during the trajectory of recurrence, and thus whether they are interchangeable or complementary; for instance, are CTCs generally present before the appearance of ctDNA or vice versa? Additionally, relationships between blood-based markers and predicted risk based upon baseline tumor genomic signatures and dynamic changes in markers with the introduction of a new therapeutic strategy are not defined, and could inform an optimal trial strategy. While there are a number of pre-analytic and analytic considerations with these tests, the field is rapidly evolving, and we anticipate that there will be a role for liquid biopsy as prognostic and predictive biomarker, given the ability to easily collect and monitor these features over time.

POTENTIAL THERAPIES FOR LATE RECURRENCE

Therapeutic intervention to reduce the risk of late recurrence of ER-positive breast cancer will require agent(s) that are effective, safe and tolerable. Several classes of agents now available and under study in advanced breast cancer and in the earlier adjuvant setting could be considered in this setting.

Inhibition of cyclin-dependent kinases 4 and 6 (CDK4/6) in combination with hormonal therapy has been a highly effective treatment regimen for metastatic HR+ breast cancer with three FDA-approved agents – abemaciclib, palbociclib and ribociclib – showing similar outcomes with somewhat differing side effect profiles (44). Randomized studies of adjuvant CDK4/6 inhibitors in early breast cancer have recently reported contrasting results, with abemaciclib reducing the risk of recurrence at 1-2 years for patients with node-positive, high-risk, ER-positive breast cancer in the MONARCH-E trial (45), and palbociclib failing to show benefit in the PALLAS (46) and PENELOPE (47) trials. Further maturation of these data and the results from a similar study of ribociclib (the NATALEE trial) will provide a better understanding of the potential efficacy of these agents early in the course of adjuvant therapy for high risk disease. In addition, correlative studies are likely to provide additional insights to guide clinical implementation or additional study. While the mixed results of adjuvant trials reported to date are somewhat disappointing, CDK4/6 inhibitors remain of interest for the prevention of late recurrence, given their proven efficacy in the advanced setting, combined with their safety and tolerability in both advanced and early stage breast cancer.

Selective Estrogen Receptor Degrader/Downregulators (SERDs) have been highly effective in pre-clinical models, with substantial, but more limited efficacy in the clinical setting likely due to the difficulty in delivering an effective dose of the sole FDA-approved injectable SERD, fulvestrant. Fulvestrant is active in tamoxifen- and aromatase inhibitor-refractory cancers and is an established treatment either alone or in combination with targeted therapies for metastatic disease. A new class of orally-bioavailable SERDs shows promise in the advanced breast cancer setting both in terms of efficacy and tolerability, and would be particularly attractive as compared to a monthly injectable agent.

Additional agents that could be considered include selective inhibitors of the PI3K/AKT/mTOR pathway, which has a well-defined role in HR+ breast cancer. Currently available agents, however, are of more limited promise for application in the late recurrence setting. The FDA-approved PI3K inhibitor, alpelisib improves disease control but not survival in PIK3CA-mutant advanced HR+ disease when added to endocrine therapy. While the availability of a predictive biomarker (PIK3CA mutation) would permit genomically-guided adjuvant therapy, drug toxicities make it an unlikely candidate for the late adjuvant setting. AKT inhibitors, currently under evaluation in Phase III trials, are also promising agents for advanced breast cancer, but side effects are also likely to be limiting.

Beyond these agents that are approved or in advanced development for ER+ breast cancer, other strategies with relevance to the biology underlying tumor dormancy and escape could have potential relevance to future late recurrence prevention efforts. Given the likely contribution of anti-tumor immunity preventing metastatic recurrences, anti-cancer immunotherapy strategies such as immune checkpoint blockade could have obvious appeal. However, to date the understanding of the role of these therapies in ER+ breast cancer is limited and given their uncommon but potentially serious toxicity and high cost, much additional work to define at risk populations and potential predictors of benefit remains before late intervention trials could be considered. With continued investigation of the biology of tumor dormancy, innovative strategies may permit future approaches for drug repurposing or novel agent development in order to specifically address dormancy or reawakening (48). Any clinical investigation of such strategies will require careful assessment of drug safety and tolerability and attention to risk stratification and patient selection.

Timing and duration of any intervention to prevent late recurrence will also require examination in order to select an effective and tolerable regimen. Because the risk reduction due to five years of anti-estrogen therapy is well-established and substantial (49), intervening just after this period is attractive

for both practical and biologic reasons. Most patients will continue through at least five years from diagnosis in the care of an oncologist, who could facilitate discussion and administration of additional treatment. Biologically, this time period may represent an increased release from dormancy at completion of the initial anti-estrogen treatment, and so an opportune time to intervene (9).

From numerous studies demonstrating that longer duration of adjuvant endocrine therapy improves outcomes, it follows that similarly cytostatic agents would require a significant duration of treatment for greatest efficacy. This must be balanced with acceptable tolerability and long-term safety for a group of patients who are otherwise clinically free from breast cancer and more than five years from initial diagnosis and treatment.

CONSIDERING THE PATIENT PERSPECTIVE

When planning any interventional trial, one must consider issues related to the patient experience that may impact decisions to participate in the trial as well as the subsequent acceptability and uptake of the intervention if clinical benefit is demonstrated. To date, little is known about how patients make decisions regarding extended adjuvant therapy nor about their experience during this phase of treatment many years after initial diagnosis.

While is it well-established that HR+ breast cancer has an ongoing risk of recurrence that extends into the second decade after diagnosis, the manner in which patients perceive their risk of recurrence many years after diagnosis has not been rigorously studied. Additionally, the risk threshold beyond which patients may be willing to consider escalation of therapy after completion of 5 years of adjuvant endocrine therapy is not known. Data collected relatively soon after diagnosis indicate that patients with breast cancer often have inaccurate perceptions of their risk of recurrence, frequently over-estimating, but sometimes under-estimating, their risk (50–52).

In the early period following diagnosis, provider communication has been shown to impact accurate perception of risk (50, 53). Optimizing strategies to communicate the risk of late recurrence after completion of 5 years of adjuvant endocrine therapy will be critical for patients considering participating in a proposed trial escalating therapy after 5 years of initial endocrine therapy and, subsequently (should the intervention succeed), when implemented in routine clinical care. Communication about biomarker results has been shown to impact patients' perception of their risk of late recurrence and subsequent decisions about extended adjuvant endocrine therapy (54). Developing strategies for communicating the prognostic and predictive information provided by biomarkers – especially for novel and potentially strongly prognostic liquid biopsy markers of MRD – in the late adjuvant setting is a priority.

The potential psychological impacts associated with fear of recurrence that may accompany a determination of being at "high risk" of recurrence justifying treatment escalation must be considered. While the association between accurate perception of risk with anxiety, distress, depression and quality of life has not been rigorously studied, it is well established that breast cancer survivors frequently experience long-lasting adverse psychological outcomes and that fear of recurrence is associated with depression, anxiety and lower quality of life (50, 55–57). For the individual patient, learning that recurrence risk remains high after completing 5 years of adjuvant endocrine therapy (in addition to their earlier primary surgery, as well as any radiation and chemotherapy) may be an unwelcome surprise. While identifying an effective therapeutic option to reduce this risk is the overall goal of late recurrence elimination strategies, it is critical that the emotional impact associated with learning about residual risk and being offered escalation of therapy so many years after diagnosis is assessed.

The degree of risk reduction conferred by a potential intervention that patients would deem worthwhile in the late adjuvant setting must also be understood. Available data suggest that many patients are willing to consider extended adjuvant endocrine therapy even if the expected risk reduction is small (56, 58). In a survey of 112 patients with stage I-III breast cancer who had completed 3-5 years of adjuvant endocrine therapy, 52% indicated they were at least "moderately" willing to consider extended endocrine therapy for only a 1% absolute reduction in the risk of recurrence, while 78% and 89% were similarly willing for expected absolute benefits of 5% and 20% respectively.

Knowledge of patient willingness to extend therapy is based on continuation of endocrine therapy with which they have personal experience, or the escalation of therapy involving addition or switching to alternative endocrine agents. In this patient population, toxicity will be an important factor when deciding to add a new treatment with different toxicities, balancing baseline risk and expected risk reduction with toxicities. Side effects such as hot flashes, sexual problems and musculoskeletal discomfort are common among patients receiving adjuvant endocrine therapy (59). The toxicity profile associated with CDK4/6 inhibitors administered in conjunction with endocrine therapy in the metastatic and adjuvant settings is generally favorable (60), though may be perceived differently in the adjuvant setting, where rates of treatment discontinuation observed in the recently reported early adjuvant trials are significant. Assessment of treatment toxicity using patient-reported outcome measures will be a key component of evaluation of a proposed treatment escalation strategy, as patient tolerance may differ in the late adjuvant setting than in the settings in which these agents have previously been evaluated. Additionally, expanding our understanding of the toxicity associated with other potential therapies, including oral SERDs discussed above, will be an important component of ongoing research.

Even very promising interventions will not successfully reduce the risk of late recurrence of HR+ breast cancer if patients do not actually take them. Not only must willingness to pursue the therapy be considered, but also adherence during therapy (taking the intervention regularly as prescribed) and persistence during therapy (not discontinuing prior to

completion of the planned course). Up to approximately 50% of patients have poor adherence to the first 5 years of adjuvant endocrine therapy or discontinue adjuvant endocrine therapy prior to completing 5 years (61). Multiple factors, such as age, beliefs about medication necessity, co-payment, side effects and more are associated with poor adherence and early discontinuation of adjuvant endocrine therapy (62–65). In trials evaluating extended adjuvant endocrine therapy, approximately 40% of participants discontinued therapy early (66). Premature treatment discontinuation in the phase III trials evaluating palbociclib and abemaciclib in the early adjuvant setting occurred in 42% and 27% of participants (45, 46), with the majority of early discontinuation due to toxicity. However, it is not known whether these findings can be extrapolated to the late adjuvant setting as tolerance may differ. Factors associated with adherence and persistence with late escalation of therapy by adding a CDK4/6 inhibitor and/or SERD must be defined. Patient tolerance and reasons for nonadherence or discontinuation can only be determined using appropriate patient reported outcome measurements. Examination of adherence as well as symptoms and side effects, quality of life, fear of recurrence, anxiety and risk perception must be assessed at regular intervals during interventional trials to determine how treatment influences adherence. These endpoints are important adjuncts to efficacy data that will inform whether escalation of therapy after 5 years with one or both of these interventions delivers clinical benefit in this setting. If late adjuvant therapies demonstrate efficacy and escalated late adjuvant therapy becomes a standard of care for patients at high risk for late recurrence, future research will need to identify potential barriers to uptake, adherence and persistence in the real-world setting.

SUMMARY

In summary, patients with a history of HR+ early breast cancer have a sustained, ongoing risk of metastatic recurrence over the course of their lifetime. Standard use of adjuvant endocrine therapy for an initial five years has modulated that risk, but it continues to persist, even with the advent of extended adjuvant therapy from years 5 - 10. New strategies are needed to reduce or eliminate this risk in order to reduce breast cancer mortality. Challenges of clinical trials in this setting include identifying patients who are ultimately destined to recur to avoid overtreatment, and identification of effective agents to successfully target the biology of potentially dormant minimal residual disease. The recent development of technologies such as tumor genomic assays that complement clinicopathologic risk assessment and blood-based assays, including CTCs and ctDNA to identify patients harboring MRD, provide an opportunity to test new therapies in this setting in patients at greatest risk. Available agents such as CDK4/6 inhibitors and oral SERDs, which have demonstrable clinical activity in advanced breast cancer, may overcome resistance to standard endocrine therapy that enables dormant tumors to emerge and spread. While these individual components provide promising tools to enable the development of late recurrence strategies, designing trials to take advantage of these advances remains challenging due to numerous unanswered questions. The prevalence of blood-based tumor markers, and their relationship with radiographically detectable disease in patients years out from treatment must be understood for the statistical design of such trials. The optimal assay(s) to measure and track MRD remain uncertain, as do the risk attributable to ctDNA and/or CTCs and lead time prior to overt metastatic disease. Most importantly, additional data to determine whether eradicating ctDNA and/or CTCs will result in reduction in recurrence and improved survival such that these assays could serve as surrogate measures is lacking. Finally, impacts on patients, ranging from the emotional impacts of diagnostic risk information to the toxicities of long-term therapy, and their resulting effects on quality of life and on treatment adherence and persistence are areas of significant uncertainty. The optimal approach to studying new agents in this setting will require incorporating patient reported outcomes assessment to understand these issues and successfully test new agents and strategies to ultimately eliminate late recurrence and death.

We have developed a collaboration spanning NCTN groups "REFINE-BrCa" (REFining Adjuvant Therapy through Identification aNd Escalation) to develop clinical trials and address existing critical knowledge gaps. An initial Phase 2 study is planned to evaluate co-primary endpoints of treatment persistence and MRD clearance in patients with high-risk ER +/HER2-negative disease upon switch to oral SERD after 4-8 years of endocrine therapy. This study will provide key data on the performance of ctDNA assays for detection and response evaluation in the extended endocrine setting. We anticipate the results of this study will rapidly inform clinical development in this field and enable the launch of a definitive Phase 3 trial (**Figure 1B**).

AUTHOR CONTRIBUTIONS

AD, DC, KK, KS, AT, HP, FZ, and PS contributed to the conceptualization and writing of the manuscript, contributing individual components and editing the whole. All authors contributed to the article and approved the submitted version.

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