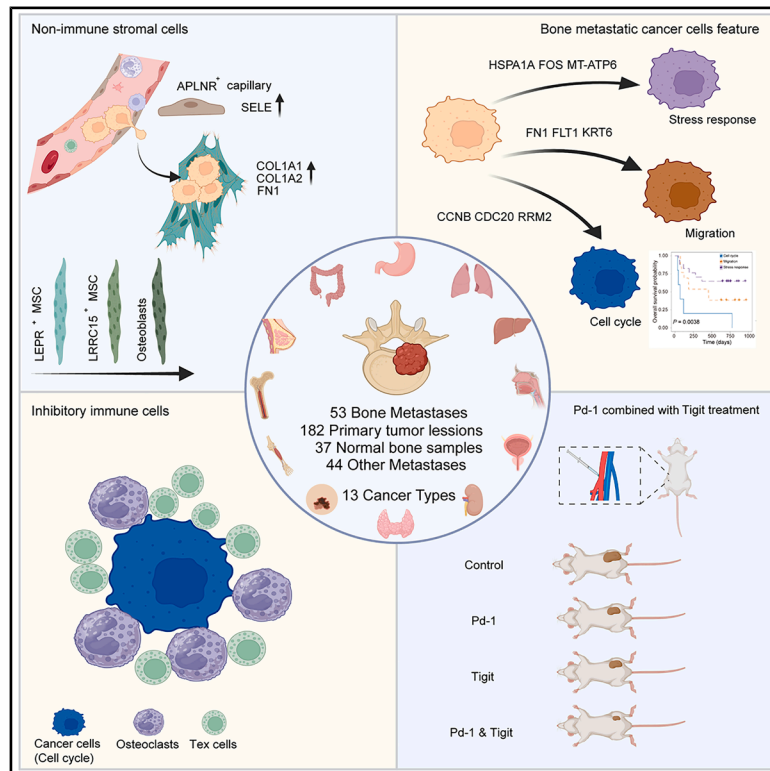


A pan-cancer single-cell transcriptomic atlas of human bone metastases

Graphical abstract



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In brief

This study constructs a pan-cancer single-cell atlas of human bone metastases, revealing niche-specific stromal and immune programs that drive tumor proliferation and immunotherapy resistance. Combined anti-PD-1/TIGIT therapy effectively suppresses tumor growth and enhances T cell cytotoxicity in mouse models.

Highlights

- A pan-cancer single-cell atlas of human spinal bone metastases
- Three cancer-cell defined functional programs associated with patient prognosis
- SELE+ capillary and osteoblasts support malignant microenvironment in bone metastases
- Combined PD-1 and TIGIT blockade restores cytotoxic immunity in bone metastases



Article

A pan-cancer single-cell transcriptomic atlas of human bone metastases

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SUMMARY

Bone is a common site for cancer metastasis, yet the mechanisms driving human spinal bone metastases (BMs) are poorly understood. Here, we obtained a single-cell RNA sequencing (scRNA-seq) atlas of 62 BMs across 13 different origins, along with paired primary tumor and normal bone marrow. Unsupervised clustering of cancer cell functional programs revealed 3 groups with distinct prognosis and tumor microenvironment. Integrative analysis with large-scale primary and metastatic pan-cancer and healthy bone marrow scRNA-seq datasets revealed *SELE*-positive endothelial cells, osteoblasts, and osteoclasts associated with cancer cell proliferation. We also identified BM-enriched exhausted CD8⁺ T cells with increased expression of immune checkpoint genes. In bone metastatic mouse models, a combined anti-PD-1/TIGIT immune therapy effectively suppressed tumor cell proliferation and significantly enhanced the cytotoxic activity of CD8⁺ T cells. Our results provide a systematic view of the molecular basis of BM and suggest future avenues for immunotherapy optimization for BM patients.

INTRODUCTION

Bone metastases (BMs) are the most common types of bone tumors in adults. It is estimated that 65%–80% of individuals with breast or prostate cancer,¹ along with 30%–60% of those with thyroid, kidney, lung, or gastrointestinal cancers,^{2,3} will experience BM. These metastases can lead to skeletal-related events, such as pathological bone fractures, pain, hypercalcemia, and compression of the spinal cord and nerves,⁴ all of which significantly impair the quality of life for patients.⁵ The principal treatments for BMs in clinical settings currently include radiotherapy, bisphosphonates, and RANKL antibodies.^{6,7} Nevertheless, a

substantial number of patients (30%–50%) still experience the development of new BMs, skeletal complications, and disease progression.¹ This underscores the urgent need for the development of new treatment options.

The spine is the most frequently affected site for BMs, comprising approximately 70% of all BMs lesions.^{8,9} Spinal metastases exhibit distinct biological and anatomical characteristics compared to those occurring in long bones. The vertebral column is distinguished by a unique venous plexus network that facilitates hematogenous dissemination,^{10,11} as well as a highly cellular marrow environment conducive to metastatic colonization.^{12,13} Clinically, spinal involvement often results in



severe pain, mechanical instability, and neurological compromise, contributing to a poorer prognosis and increased functional disability.¹⁴ Although a recent study has profiled BMs across multiple cancer types at the single-cell level, it predominantly focused on metastases occurring in the limbs or long bones.¹⁵ In contrast, studies specifically addressing spinal vertebral metastatic lesions remain scarce. Such investigations are urgently needed to elucidate the unique features of spinal metastases.

BMs are composed of multiple elements, including tumor cells, endothelial cells (ECs), osteoclasts, osteoblasts, mesenchymal stromal cells, myeloid cells, lymphatic immune cells, and bone matrix.^{16,17} This complex microenvironment makes treating BMs a significant challenge in the clinical setting. Recent studies have shown that the bone microenvironment might augment cancer cells to further secondary metastasis.^{13,18} However, the reasons behind BMs vary greatly due to tumor heterogeneity, involving factors like the epithelial-mesenchymal transition (EMT) and chemokines.^{19,20} Interestingly, despite these differences, the cellular interactions within the microenvironments of various BMs share many similarities, aspects that remain murky and warrant more in-depth investigation.

Recent advancements in high-throughput single-cell sequencing (scRNA-seq) have delved into a variety of primary tumors, thereby improving our understanding of the tumor microenvironment.^{21–24} However, single-cell analysis focusing on BMs across various cancer types is lacking. In this study, we systematically collected bone metastatic tumor tissues from 13 different primary cancer types, predominantly derived from spinal lesions, together with available paired primary and adjacent normal bone tissues. Utilizing these samples, we conducted scRNA-seq to examine the composition and interplay among tumor, immune, and stromal cells within the complex microenvironment of BMs. The current study will enhance our understanding of BMs and offer potential therapeutic strategies for the disease.

RESULTS

Single-cell transcriptomic landscape of pan-cancer BMs

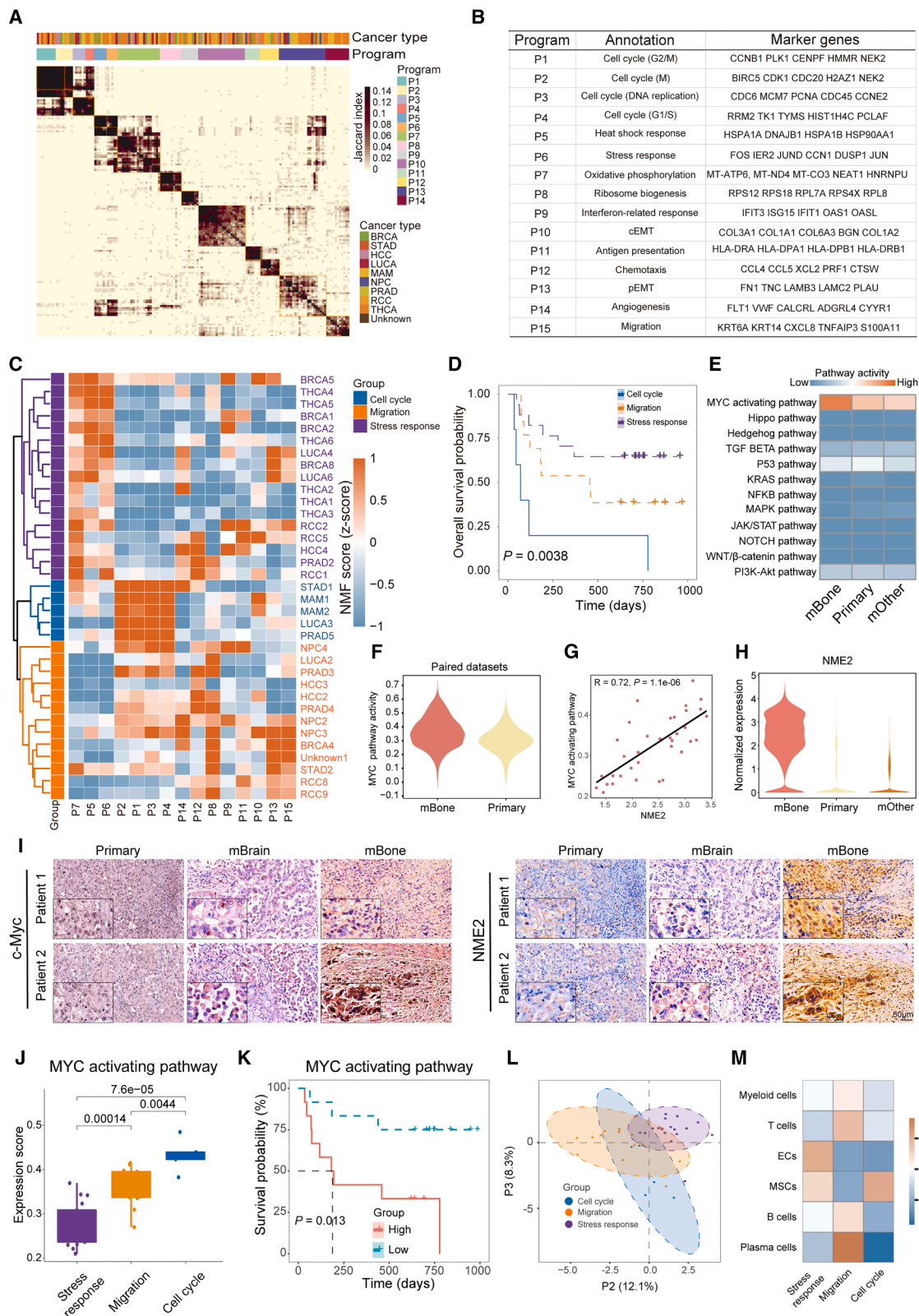
To generate a comprehensive single-cell transcriptome atlas of BMs, we performed scRNA-seq on 53 fresh bone metastatic

specimens, of which 43 were from spinal metastases. The samples were collected from 52 patients diagnosed with one of the 13 cancer types, including breast carcinoma (BRCA) ($n = 8$), colon adenocarcinoma (COAD) ($n = 3$), hepatocellular carcinoma (HCC) ($n = 4$), lung carcinoma (LUCA) ($n = 6$), malignant melanoma (MAM) ($n = 2$), nasopharyngeal carcinoma (NPC) ($n = 3$), prostate adenocarcinoma (PRAD) ($n = 5$), renal cell carcinoma (RCC) ($n = 9$), stomach adenocarcinoma ($n = 2$), thyroid carcinoma (THCA) ($n = 6$), and unknown primary carcinoma ($n = 1$), as well as plasmacytoma ($n = 3$) and one case of myofibroblastoma. In addition, we collected 6 paired primary (1 LUCA and 2 PRAD) and spinal metastatic samples as well as 4 paired spinal metastases and adjacent normal bone samples (2 LUCA) from the same patients for scRNA-seq analysis. Six other paired primary and metastatic (3 LUCA) samples²⁵ as well as 49 paired BMs and adjacent normal bone or bone marrow samples from recently published studies^{26,27} were also included in our study (Figures 1A and 1B; clinical characteristics are listed in Table S1). Meanwhile, we retrieved 12 additional scRNA-seq datasets^{23,28–38} of 182 primary cancer samples, 2 datasets^{39,40} of 37 healthy bone marrow tissues, and 8 datasets^{30–33,41,42} of 44 other metastatic samples from previous studies for comparison with BMs (see key resources table). After stringent quality control, a total of 1,304,048 cells collected from the BMs (430,100 cells) (Table S2), normal bone marrows (178,512 cells), primary tumors (414,906 cells), and other metastatic tumors (280,530 cells) were used for the following analysis.

Using a customized computational pipeline, we first performed cell clustering analysis and identified 282,266 malignant cells and 926,013 stromal cells (Figure 1C). Unlike stromal cells, malignant cells formed independent clusters corresponding to individual samples, indicating the high degree of inter-tumor heterogeneity (Figure S1A and S1B), consistent with previous reports.^{43,44} In addition, malignant cells derived from epithelial cells showed high expression of *KRT18*, melanocyte-derived cancer cells highly expressed *MLANA*, and plasmacytoma cancer cells highly expressed *MZB1* (Figure S1C). Plasmacytoma cancer cells were distinguished from plasma cells based on the copy number variation (CNV) profiles using the inferCNV method, where cancer cells showed high CNVs (Figure S1D).

Figure 1. Single-cell transcriptome atlas of pan-cancer bone metastases

(A) Bone metastases atlas and schematic overview of the study. (Upper) The number of samples from bone metastatic atlas and public datasets of primary cancers, normal bone marrows, and other metastatic tissues included in this study. The origin primary cancer types of bone metastases were shown in the diagram. The metastatic sites for other metastatic samples were shown. (Lower left) Number of samples and origin tissues of paired samples included in the study. (Lower right) Schematic diagram of the study. Single cells were dissected from bone metastatic samples and paired primary or normal bone marrow tissues and following single-cell transcriptomic profiling. Adjacent tissues from the same samples were paraffin fixed and used for immunostaining analysis. (B) Nightingale Rose plots showing the number of cells collected in each cancer type from bone metastases, primary or other metastatic tissues, and normal bone marrows. Cell numbers collected from paired bone metastases and normal bone tissues as well as paired bone metastases and primary tumor tissues were shown separately below. The height of the red, blue, yellow, and orange bands indicates the number of cells from the bone metastatic, normal bone, primary, and other metastatic tissues, respectively. (C) Uniform manifold approximation and projection (UMAP) visualization of major cell types detected in the integrated datasets of bone metastases, normal bone tissues, primary, and other metastatic tumors. Cells are colored by cell types labeled by legend. (D) Bubble plot showing marker gene expression levels across defined clusters in the cohort. Dot size indicates fraction of cells with expression of the indicated genes, and color from red to gray indicates a high to low normalized expression. (E) Bar plots displaying relative cellular fractions of cancer cells and non-malignant cells across bone metastatic samples. Gray bar indicates proportion of cancer cells. Pink bar indicates proportion of non-malignant cells. (F) Representative examples of bone metastatic tissues stained by IHC with anti-KRT19 (brown) antibodies. Scale bar, 100 μ m. (G) Boxplot showing the compositions of non-malignant cells in bone metastatic samples ($N = 53$) among different cancer types ($N = 53$). Box represents the first and third quantiles, and whiskers indicate maximum and minimum values. The p value was calculated using the Kruskal-Wallis test.



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Among the stromal cells, ECs were characterized by specific high expression of *PLVAP* and *PECAM1*, mesenchymal stromal cells were characterized by *THY1* and *CTHRC1*, and immune cells clusters were divided into myeloid cells (*LYZ* and *AIF1*), T/NK cells (*CD3D* and *NKG7*), mast cells (*TPSB2* and *TPSAB1*), plasma (*MZB1* and *JCHAIN*), and B cells (*MS4A1* and *CD79A*) in the integrated dataset (Figure 1D). Immune cells expressed lower numbers of genes with comparable RNA counts in comparison with other cell types (Figure S1E). Furthermore, the proportion of malignant cells showed high variability among bone metastatic samples, ranging from 0.1% to 80.4% (Figure 1E), with a median of 32.7% across biopsies, which was further confirmed by immunostaining for KRT19 in bone metastatic samples (Figure 1F).

Despite the existence of stromal cells in various cancer types, the composition of stromal cells varied significantly among different cancer types. BMs from COAD and myofibroblastoma showed the highest infiltration of stromal cells and those from plasmacytoma and MAM had the lowest level of stromal cells (Figures 1G and S1F). For stromal cell types, we found that ECs were most abundant in PRAD and THCA, whereas T/NK cells were more abundant in NPC, COAD, HCC, RCC, and myofibroblastoma (Figure S1F). In addition, comparative analysis of major cell types between BMs and normal bone tissues showed that endothelial and mesenchymal stromal cells were significantly enriched in BMs (Figure S1G). These results indicated the cellular heterogeneity in bone metastatic niche of different cancer types that needs to be fully characterized.

Transcriptional programs in bone metastatic cancer cells

To characterize the molecular features of malignant cells in BMs, we next compared the gene expression profiles of cancer cells across different tissues. To ensure that malignant cells were free of bone cell contamination, logistic regression analysis was performed against bone and mesenchymal stromal

cells using a recently published normal bone marrow scRNA-seq dataset⁴⁵ (Figure S2A), and cells predicted as bone cells or mesenchymal stromal cells were removed from the following analysis (see STAR methods). Besides, the malignant cells were also validated by high CNVs in comparison with stromal cells (Figure S2B). Given the striking heterogeneity of malignant cells among individuals (Figure 1C), we next exploited non-negative matrix factorization (NMF) to identify transcriptional programs associated with BMs shared by diverse primary cancer types in our pan-cancer dataset following recent studies.^{41,46,47} The consensus transcriptional programs across different tumor samples were identified by hierarchical clustering of expression profiles of coherent genes (Figures 2A and S2C). Using this procedure, we identified a total of 15 consensus programs (P1–P15) in our dataset, all of which were found in multiple or all bone metastatic samples (Figures 2B and S2D).

The 15 consensus programs were annotated based on the cellular processes of the top expressed genes in each consensus program (Figure 2B). Correlation analysis among the consensus programs revealed 3 distinct clusters (Figure S2E). Cluster 1 (P1, P2, P3, and P4) was annotated as cell cycle-related programs. P1 was characterized by G2/M-phase genes (e.g., *CCNB1*, *CENPF*, *HMMR*, *NEK2*, and *PLK1*), P2 highly expressed M-phase genes (e.g., *BIRC5*, *CDK1*, *CDC20*, *CENPF*, *H2AZ1*, *NEK2*, *PTTG1*, *TUBA1B*, *UBE2C*, *UBE2S*, and *NUF2*), and P3 and P4 were enriched in DNA replication- (e.g., *CDC6*, *MCM7*, *PCNA*, *CDC45*, *CCNE2*, *GIN52*, *MCM10*, and *CDT1*) and G1/S phase- (e.g., *RRM2*, *TK1*, and *TYMS*) associated genes, respectively. The cluster 1 programs were identified in 18 bone metastatic samples from 8 different primary sites (Figures S2D and S2E). The cluster 2 programs (P5, P6, and P7) were associated with Stress response (Figures 2B and S2E). P5 was characterized by genes of heat shock response (e.g., *HSPA1A*, *DNAJB1*, *HSPA1B*, and *HSP90AA1*). The heat shock proteins were reported to increase overall stress tolerance and improve angiogenesis.⁴⁸ P6 highly expressed genes of Fos and Jun families

Figure 2. Features of cancer cells revealed 3 groups of bone metastatic samples

- Heatmap showing similarities between NMF clusters identified across metastatic cancer cells. Programs were grouped by hierarchical cluster. Color from black to yellow indicates a high to low Jaccard index.
- Annotation and selected top genes for each program.
- Heatmap showing mean NMF score of programs in each sample. Hierarchical clustering samples using mean NMF score of programs. Color from orange to blue indicates a high to low NMF score.
- Kaplan-Meier plots showing worse clinical outcome in bone metastatic samples with Cell cycle group. *p* value was calculated by log rank test.
- Heatmap showing the activity of oncogenic signaling pathways across bone metastases, primary, and other metastatic tissues. Color from orange to blue indicates a high to low pathway activity.
- Violin plot showing MYC pathway activity in cancer cells from paired bone metastases and primary tumors. The pathway activity was calculated as the averaged expression level of all genes involved in the MYC signaling pathway.
- The scatterplot showing the correlation between *NME2* expression and MYC pathway activity in tumor cells from bone metastatic lesions.
- Violin plot showing the expression of *NME2* in cancer cells between bone metastatic and primary tumors as well as other metastatic cancers.
- Immunohistochemical staining of c-Myc and *NME2* in matched primary tumors, brain metastases (mBrain), and bone metastases (mBone) from 2 patients. Both proteins showed higher expression in bone metastases. Insets show higher-magnification views of the boxed regions. Scale bars: 50 μ m for low-magnification images and 10 μ m for insets.
- Boxplot showing the enrichment scores of the MYC-activating pathway across 3 cancer groups (Stress response: *n* = 17, Migration: *n* = 13, Cell cycle: *n* = 5). *p* value was calculated by Kruskal-Wallis test.
- Kaplan-Meier curves showing worse clinical outcome of BM patients with high level of MYC pathway activity in cancer cells. *p* values were calculated by log rank test.
- Principal-component analysis of cancer cell program features expression in each sample. Dots are colored by cancer groups.
- Heatmap showing the relative enrichment of major immune and stromal cell types across the 3 cancer groups. Color from orange to blue indicates a high to low relative enrichment score.

(e.g., *FOS*, *IER2*, *JUND*, *CCN1*, *DUSP1*, and *JUN*), which were shown to be engaged in stress response.^{47,49} P7 was enriched for genes associated with oxidative phosphorylation (e.g., *ATP6*, *COX1*, *COX2*, *CYTB*, *ND1*, and *ND2*). Oxidative phosphorylation may target and eradicate cancer stem cells, thereby slowing down the development of drug resistance.^{50,51} In addition, 8 programs (P8–P15) belonging to cluster 3 were enriched with Migration-related genes. For example, P10 was related to a complete mesenchymal module (cEMT) and P13 was enriched for genes associated with integrin1 pathway and cell focal adhesion (e.g., *FN1*, *TNC*, *LAMB3*, *LAMC2*, and *PLAU*), corresponding to partial mesenchymal module (pEMT).^{47,52} P14 highly expressed genes associated with tumor angiogenesis (e.g., *VWF*, *CALCRL*, and *FLT1*), which has been reported to promote tumor progression and metastasis in a variety of tumors.^{53–55} P15 was found in 31 of 33 samples and was characterized by high expression of epithelial cell markers (*KRT6A* and *KRT14*) and genes involved in cell migration regulation (*CXCL8*, *TNFAIP3*, *S100A11*, and *TMSB4X*).

The identification of 15 tumor programs across different tumor origins indicated the heterogeneity in the bone metastatic environment. Then we performed hierarchical clustering of the bone metastatic samples in our cohort based on their relative expression level of the 15 tumor programs and identified 3 groups with distinct characteristics of tumor profiles (Figure 2C). Group 1 showed increased abundance of cluster 1 consensus programs (P1–P4) and was thus named as Cell cycle group, Group 2 (Stress response group) composed of 13 samples highly expressed cluster 2 (P5, P6, and P7) programs, which corresponded to Stress response. Group 3 included 17 samples displaying high level of cluster 3 consensus programs and was named as Migration group. To demonstrate the applicability of our single-cell transcriptome-based classification of bone metastatic tumors, we further applied the tumor programs to classify the 71 bone metastatic samples in the MET500 cohort,⁵⁶ and the classification resulted in 3 groups similar to that found in our single-cell transcriptome dataset (Figures S2F–S2H). Notably, the 3 groups had different survival probabilities ($p = 0.038$), with the Cell cycle group showing the worst survival probability (Figure 2D).

To understand whether the molecular features of bone metastatic cancer cells were inherited from their primary tumors, we next compared the abundance of tumor programs in paired primary and BM samples from the same patients. The results showed that all these 15 tumor programs existed in the primary tumors (Figure S2I), but the Cell cycle-related programs exhibited higher level in BMs than in primary tumors (Figure S2J), indicating that cancer cells in BMs might inherit the features of cancer cells in the primary sites. To further explore the change of cancer cells from primary tissues to BMs, we performed trajectory analysis for cancer cells from paired primary and bone metastatic samples and found that cancer cells from the primary site were differentiated into those in the bone metastatic sites (Figure S2K). Genes upregulated in bone metastatic tumors compared to primary tumors were significantly enriched in MYC activating and regulation of leukocyte migration pathway (Figures S2L and S2M). We also examined the activity of oncogenic pathways between BMs and primary tumors or other metastatic tissues and found that MYC signaling pathway showed

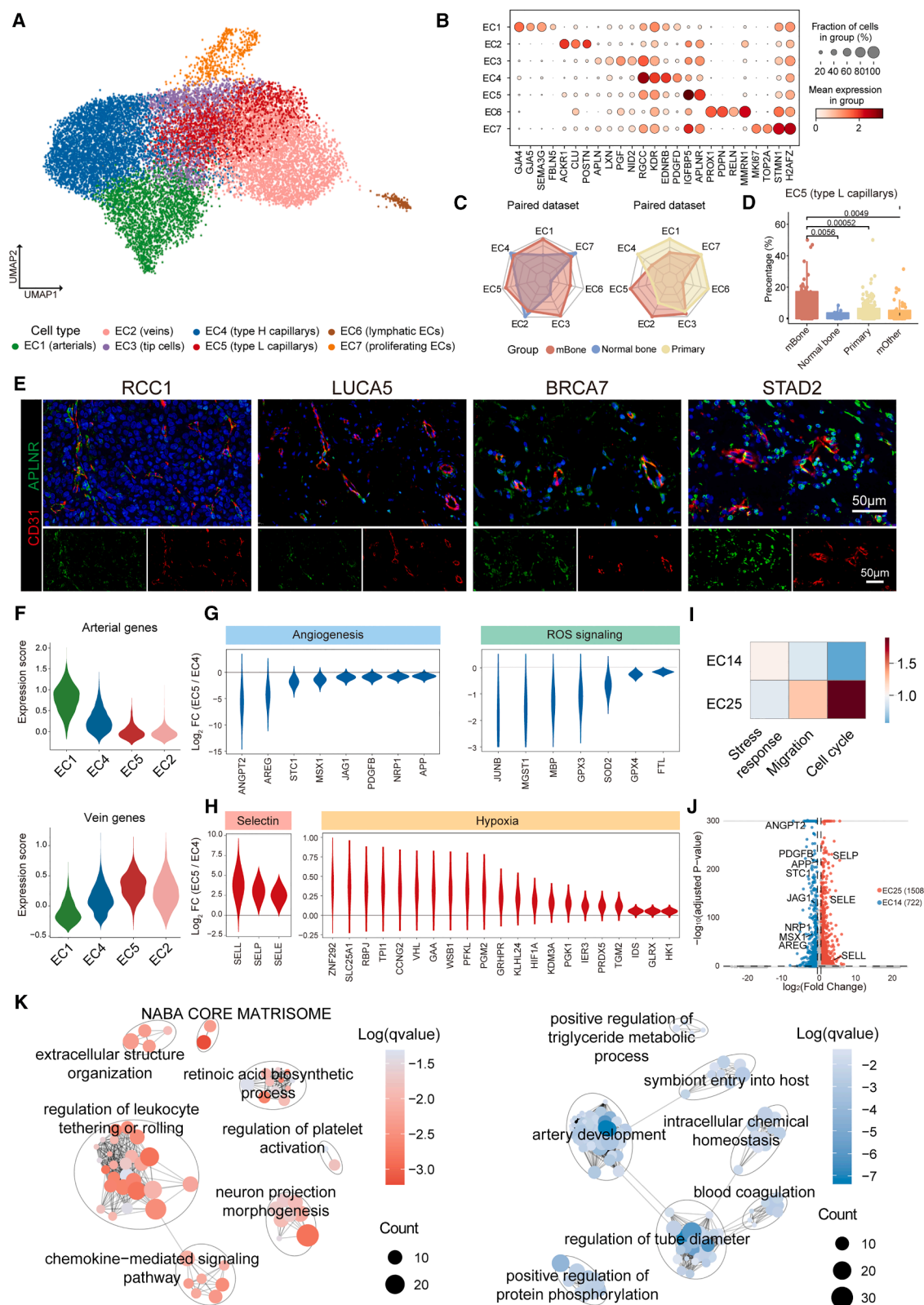
the highest activity in BMs than other tissues (Figure 2E). The MYC pathway activity was also significantly upregulated in BMs in comparison with paired primary tumor tissues (Figure 2F).

The MYC pathway is one of key signaling pathways in tumor initiation and progression, and its aberrant activation is closely associated with various malignancies.⁵⁷ Previous studies have shown that NME2 (NM23-H2/NDPK-B) functions as the purine-binding factor (PuF) that directly engages with the NHE III1 region of the MYC promoter.^{58–60} We found that the NME2 expression was significantly positively correlated with the MYC signaling pathway score in BMs (Figure 2G). Furthermore, NME2 was increasingly expressed along the trajectory (Figure S2L) and was specifically highly expressed in BMs (Figures 2H and S2N). Immunostaining analysis confirmed the higher level of c-MYC and NME2 expression in BMs, with the latter barely expressed in other tissues (Figure 2I). The BM-enriched NME2 gene expression and MYC pathway activity also showed significantly higher levels in Cell cycle and Migration groups than Stress response group (Figures 2J and S2O). Higher level of NME2 expression and MYC pathway activity was associated with poorer survival of bone metastatic patients (Figures 2K and S2P). Together, these results indicated that cancer cells disseminated into the bone metastatic tissues showed different molecular features from those of primary tumors or other metastatic tumors.

Interestingly, principal-component analysis based on the composition of all stromal and immune cell subtypes also revealed 3 clusters corresponding to the 3 cancer cell-classified patient groups (Figure 2L). For example, mesenchymal stromal cells were enriched in the Cell cycle group and all immune cells (myeloid, T, B and plasma cells) were more abundant in the Migration group (Figure 2M), suggesting the potential crosstalk between the tumor microenvironment and the heterogeneity of cancer cells in the bone microenvironment.

Molecular diversification of ECs in the BM

Given the highly vascularized feature of bone marrow, we next examined the role of ECs in the microenvironment of BMs. Recent studies have revealed that ECs in the BM exhibited molecular and functional diversity, which contributed to the regulation and maintenance of the bone marrow microenvironment.^{61–63} Thus, understanding the molecular diversification of ECs in the bone metastatic environment is crucial for unraveling the complex cellular interactions and regulatory mechanisms that govern the BM of diverse primary cancers. Characterization of the molecular landscape of 15,885 ECs in the BM environment revealed 7 clusters of ECs with distinct gene expression profiles by unsupervised clustering (Figures 3A and S3A). EC1 highly expressed genes of arterial markers (e.g., *GJA4*, *GJA5*, *FBLN5*, and *SEMA3G*).^{41,64} EC2 highly expressed markers including *ACKR1*, *CLU*, and *POSTN*, acting as key factors for venous cells.⁴¹ EC3 was designated as tip-like cells with high expression of *APLN*, *LUX*, *PGF*, and *NID2*.^{65–67} EC4 and EC5 clusters were defined as capillary cells by high expression of *RGCC* and *KDR*.⁶⁸ EC6 and EC7 were annotated as lymphatic EC and proliferating EC, respectively, by their corresponding marker genes (Figure 3B). The proportion of EC clusters varied among different cancer types, with EC4 showing the highest proportion in THCA (Figure S3B).



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We then compared the composition of ECs between paired BMs and normal bone marrows, and found that EC1, EC3, and EC5 were more abundant in BMs (Figure 3C). Further comparison with paired primary tumors showed that the proportion of EC5 remained to be higher in BM. A consistent higher abundance of EC5 was observed in BMs compared with the larger number of samples from primary tumors, normal bone marrows, and other metastatic tissues with statistical significance (Figure 3D). Further immunofluorescence staining assay validated the general existence of EC5 cells in bone metastatic tissues (Figures 3E and S3C). In comparison with the high expression of arterial genes in EC4, EC5 highly expressed vein-associated genes (Figure 3F). Functional enrichment of marker genes showed that EC4 was associated with epithelial cell proliferation and regulation of cell adhesion, whereas EC5 was correlated to cell tethering or rolling and cell surface interactions at the vascular wall (Figure S3D).

Specifically, we found that the arterial capillary EC4 highly expressed genes involved in reactive oxygen species signaling and angiogenesis (e.g., *PDGFB*, *ANGPT2*, and *AREG*)⁶⁹ (Figure 3G), which was related to the formation of blood vessels and growth of tumor cells.^{70,71} Consistently, our results showed that the proportion of EC4 was positively correlated with the proportion of tumor cells in the bone metastatic tissues ($R = 0.43$; $p = 0.0012$; Figure S3E). In contrast, hypoxia-associated genes were highly expressed in EC5 (Figure 3H), indicating the hypoxia environment in the vein-like capillary cells. Interestingly, selectin genes (*SELE*, *SELL*, and *SELP*), which mediated tumor cells into and out of blood vessels,^{72–75} were highly expressed in EC5 (vein-like capillary) and EC2 (veins) (Figures 3H and S3F). E-selectin expressed within the bone vascular niche has been demonstrated to reprogram disseminated cancer cells by inducing mesenchymal-epithelial transition (MET) and activating Wnt signaling pathway, thus promoting bone colonization.⁷⁶ The protein expression of SELE in the bone metastatic tissues was also validated by immunofluorescence staining assay (Figure S3G). In addition, the *SELE*-positive EC2 and EC5 cells were enriched in the Cell cycle group derived from tumor cell programs (Figure 3I). Given the poor prognosis of patients in the Cell cycle group, we

hypothesized that the high *SELE* expression in veins and vein-like capillary cells might serve as an adhesion and trafficking interface that may facilitate tumor-endothelial interactions.

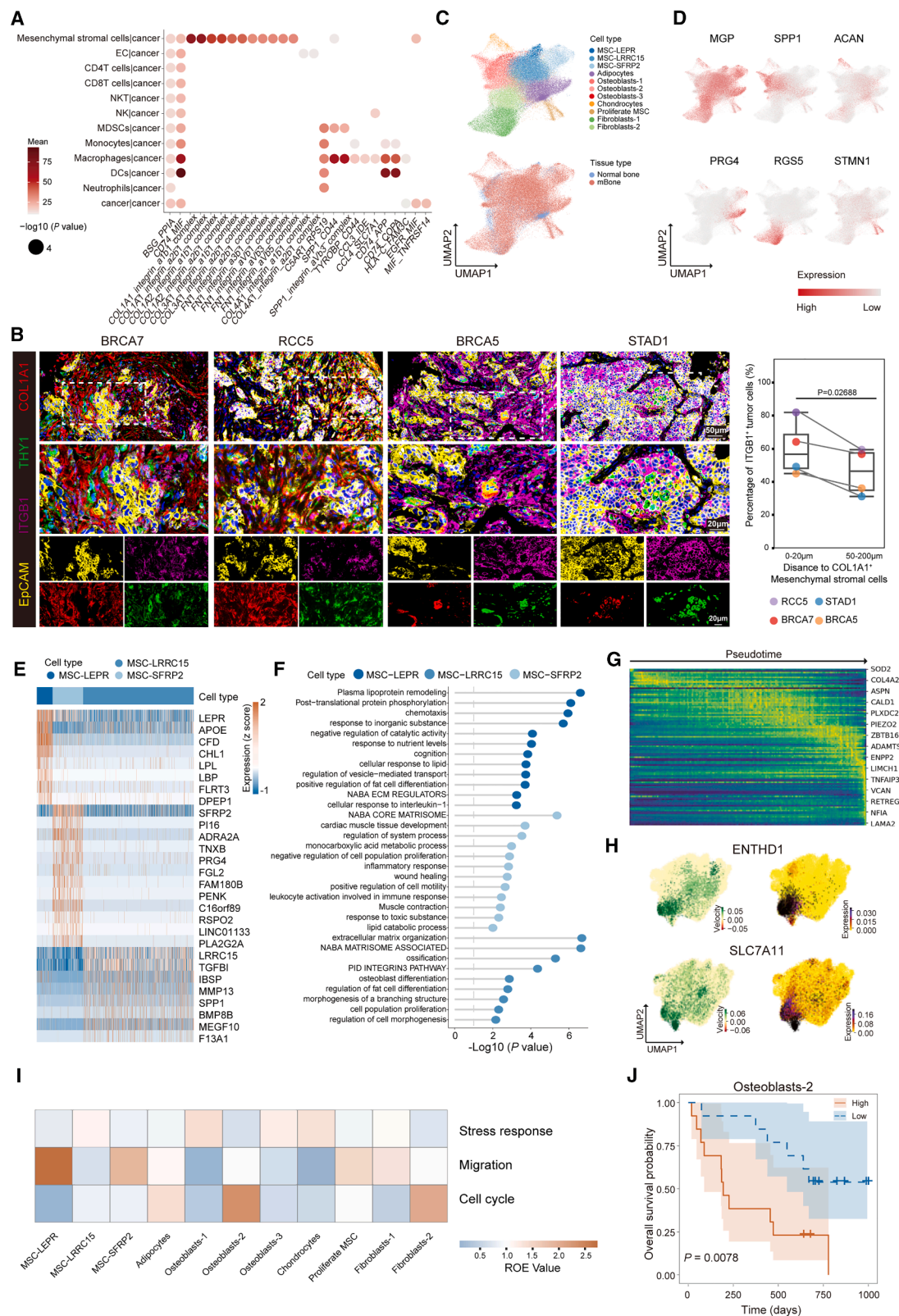
Therefore, we next performed differential gene expression analysis between *SELE*-positive (EC2 and EC5) and *SELE*-negative (EC1 and EC4) ECs and identified 1,508 differentially expressed genes (Figure 3J). Genes highly expressed in *SELE*-positive ECs were enriched in pathways including chemokine-mediated signaling, extracellular structure organization, and regulation of leukocyte tethering or rolling. In contrast, *SELE*-negative cells showed pathway clusters related to artery development, positive regulation of protein phosphorylation, and positive regulation of triglyceride metabolic process (Figure 3K). To examine tumor-related molecular interactions enriched in *SELE*-positive ECs, we further performed ligand-receptor analysis between EC subpopulations and cancer cells using CellPhoneDB.⁷⁷ The results showed that *SELE*-positive ECs and cancer cells from the Cell cycle group exhibited the strongest interaction strength through TIMP1-CD63 pairs (Figure S3H). In combination with the high expression of hypoxia-related features in *SELE*-positive ECs, this result was consistent with a previous study showing that hypoxia signaling pathway can induce *Timp1* expression via HIF1 α activation.⁷⁸ Together, these results indicated the heterogeneity of ECs and the interaction of *SELE*-expressing veins and vein-like capillary cells with proliferative cancer cells in the bone metastatic environment.

Heterogeneity of mesenchymal stromal cells and their differentiation in BMs

Mesenchymal stromal cells constitute one of the most important components in the bone marrow environment.^{79,80} In order to understand the role of mesenchymal stromal cells in the context of BMs, we identified 61,703 mesenchymal stromal cells based on the expression of classical marker gene *THY1* (Figure 1D). Cell-cell interaction analysis between different stromal cell types and cancer cells showed that mesenchymal stromal cells demonstrated the strongest interaction with cancer cells in BMs (Figure 4A). The interaction was mediated by the bonding of type I collagen or fibronectin on the mesenchymal stromal cells

Figure 3. Subtypes of ECs in pan-cancer bone metastases

- (A) UMAP visualizations of EC subtypes. Cells are colored by cell types labeled by legend.
- (B) Bubble plot showing expression levels of selected signature genes for EC subtypes. Dot size indicates fraction of cells with expression of the indicated gene, and color from red to white indicates a high to low normalized expression.
- (C) Radar chart showing the infiltration proportions of EC subtypes in paired bone metastatic and normal bone marrow samples (first column) and in paired primary and bone metastatic samples (second column). The radius was individually normalized for each cell subtype with the highest abundance of the cell subtype in different tissues.
- (D) Boxplot showing the percentage of indicated cell type EC5 among all samples from bone metastases ($n = 53$), normal bone marrows ($n = 12$), primary tumors ($n = 138$), and other metastatic tissues ($n = 33$) included in this study.
- (E) Representative images of BM tissues stained by multiplex IHC (mIHC) with anti-CD31 (red) and APLNR (green) antibodies. Scale bars, 50 μ m.
- (F) Violin plots showing expression scores of arterial associated genes and vein associated genes across EC subtypes. Violins are colored by cell types labeled by x axis.
- (G and H) Violin plots showing fold change (log2FC) of the different expression genes between EC4 and EC5. Violin plots showing genes highly expressed in EC4 (G) and EC5 (H), respectively. Titles show the pathway in which genes on x axis are involved.
- (I) Heatmap showing the relative enrichment of EC14 (EC1 and EC4) and EC25 (EC2 and EC5) across the 3 cancer groups. Color from red to blue indicates a high to low relative enrichment score.
- (J) The volcano plot displays differentially expressed genes between EC14 (EC1 and EC4) and EC25 (EC2 and EC5). Red dots represent genes upregulated in EC25, while blue dots represent genes upregulated in EC14.
- (K) Enrichment network plots of upregulated pathways in *SELE*-positive (red, left) and *SELE*-negative (blue, right), generated with aPEAR. Node size indicates the number of genes enriched per pathway. The color reflects log-transformed q values. Functionally related pathways are grouped by gene overlap.



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and integrin on the cancer cells. Immunostaining assay subsequent to quantitative analysis showed that mesenchymal stromal cells (expressing *COL1A1*) and cancer cells (expressing *ITGB1*) were colocalized in close proximity in the bone metastatic tumors (Figure 4B). Besides, we found that collagen genes showed higher expression in mesenchymal stromal cells of BMs than healthy bone marrows and integrin-related genes also exhibited higher expression level in bone metastatic cancer cells than that in primary cancers (Figure S4A). The type I collagen has been reported to be associated with the malignant progression of various cancers.^{81,82} These results suggested that mesenchymal stromal cells might play an important role in the BMs. Sub-clustering analysis of mesenchymal stromal cells revealed 2 fibroblasts, 3 mesenchymal stem cells (MSCs), 3 osteoblasts, 1 adipocyte, 1 chondrocyte, and 1 proliferate mesenchymal stromal cell subtypes with corresponding marker genes (Figures 4C, 4D, and S4B). These mesenchymal stromal cell subtypes were distributed in diverse cancer types with differential abundances (Figure S4C). THCA and MAM contained a higher frequency of fibroblast cells, whereas osteoblasts showed higher proportions in BRCA, COAD, LUCA, and NPC (Figure S4D).

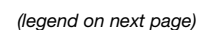
The MSCs constituted nearly 50% of all mesenchymal stromal cells for most cancer types except for PRAD and RCC (Figure S4D). Among the 3 MSC subtypes, MSC-LEPR highly expressed *CFD*, *APOE*, *LPL*, *CHL1*, and *FLRT3*, associated with plasma lipoprotein remodeling and chemotaxis. MSC-SFRP2 showed high expression levels of *ADRA2A*, *CLIC2*, and *PI16*, participating in the regulation of muscle system process and inflammatory response. MSC-LRRC15 highly expressed genes involved in extracellular matrix remodeling and ossification, including *IBSP* and *MMP13* (Figures 4E and 4F). Different characteristics of MSC-like cells imply different functions in the BM and differentiation process. For example, *CXCL12*, a bone metastatic chemokine,⁸³ was highly expressed in MSC-LEPR (Figure S4E). Further examination showed that this gene was also highly expressed in normal bone marrow tissues (Figure S4F). In addition, we found that MSC-LEPR was more abundant in paired normal bone marrows than in BMs (Figure S4G), in line with the preferential distribution of LEPR⁺ MSCs in the endosteal versus medullary regions in the bone marrow.

Diffusion map analysis revealed a differentiation trajectory of mesenchymal stromal cells from MSCs to osteoblast-like, chondrocyte-like, and adipocyte-like cells and fibroblasts (Figure S4H). Osteoblasts and fibroblasts exhibited significant enrichment in BMs than normal bone marrows (Figure S4I). Osteogenic-like cells could be further divided into 3 subtypes based on the heterogeneity in gene expression patterns (Figures 4C and S4B). Trajectory analysis revealed a differentiation path from MSC to osteoblast-like cells (Figures 4G and S4J), indicating the contribution of MSCs to the osteogenesis. Specifically, MSC-LRRC15, the more abundant cell type in BMs (Figure S4G), turns out to be the osteoprogenitor state according to trajectory of osteogenesis (Figure S4J), consistent with the ossification function of MSC-LRRC15 (Figure 4F). Genes associated with osteogenic differentiation (*SPP1* and *BMP8B*) were highly expressed by MSC-LRRC15 (Figure 4E). *MMP13*, another marker gene of MSC-LRRC15, was reported to be upregulated in osteoprogenitors under the effect of tumor cell secretory factors.⁸⁴ In addition, *APELA* and *LRRC15* were robustly expressed in MSC-LRRC15 (Figure S4K). *APELA* activates ERK signaling,⁸⁵ which subsequently upregulates the expression of osteogenic transcription factors such as *RUNX2*.⁸⁶ *LRRC15* can regulate the osteogenic differentiation of MSCs by modulating the NF- κ B signaling pathway.⁸⁷

As for osteoblasts-like cell subsets, osteoblasts-1 highly expressed genes associated with ossification and osteoblast differentiation and osteoblasts-2 displayed a hypoxic phenotype with high expression of genes involved in HIF1 pathway, whereas interferon (IFN) signaling-associated genes were highly expressed in osteoblasts-3 (Figure S4L). Among them, osteoblasts-2 tended to be the end-state of ossification (Figure S4J). *ENTHD1* and *SLC7A11* were highly expressed in osteoblasts-2 (Figure 4H), indicating their important roles during the osteogenic development in the bone metastatic microenvironment. Additionally, cell-cell interaction analysis between osteoblasts-2 with cancer cells revealed TIMP1-CD63 as the strongest interaction (Figure S4M). The TIMP1-CD63 signaling axis plays a key role in tumor progression and immune evasion, and its high activity is closely associated with poor prognosis in pancreatic ductal adenocarcinoma.⁸⁸ Consistently, the osteoblasts-2 was found to be enriched in the

Figure 4. Characterization of mesenchymal stromal cells subtypes

- (A) Bubble plot showing interaction pairs between cancer cells and stromal cells. Color from red to white indicates a high to low average of interaction intensity.
- (B) (Left) Representative mIHC images of bone metastases showing the distribution of ITGB1⁺ tumor cells (ITGB1⁺EpCAM⁺) around COL1A1⁺ mesenchymal stromal cells (COL1A1⁺ THY1⁺). EpCAM (yellow), ITGB1 (magenta), THY1 (green), and COL1A1 (red). Scale bars: 50 μ m (top) and 20 μ m (bottom). (Right) Boxplot showing the percentage of ITGB1⁺ tumor cells at different distances to COL1A1⁺ mesenchymal stromal cells ($n = 4$). Two-sided paired t test.
- (C) UMAP visualizations of mesenchymal stromal cells subtypes and their tissue origins. Cells are colored by cell types (upper) and by tissue types (lower).
- (D) UMAP visualization showing expression of selected marker genes for the major cell lineages. Mesenchymal stromal cells: *MGP*; osteogenic cells: *SPP1*; chondrogenic cells: *ACAN*; adipogenic cells: *PRG4*; fibroblasts: *RGS5*; proliferate MSC: *STMN1*. Color from red to gray indicates a high to low normalized expression.
- (E) Heatmap showing marker genes for MSC-like cell subtypes MSC-LEPR, MSC-SFRP2, and MSC-LRRC15. Cells are ordered by cell type labeled on legend. Color from orange to blue indicates a high to low normalized expression.
- (F) Lollipop plot showing representative functional enriched terms in MSC-LEPR, MSC-SFRP2, and MSC-LRRC15 cell subtypes.
- (G) Heatmap showing gene expression in MSC subtypes and osteoblasts subtypes over pseudotime.
- (H) UMAP visualization showing the velocity (left) and expression (right) levels of genes highly expressed on osteoblasts-2. Color from green to red indicates a high to low velocity. Color from black to yellow indicates a high to low normalized expression.
- (I) Heatmap showing the relative enrichment of stromal cell subsets across the 3 cancer groups (Stress response, Migration, and Cell cycle). Color from orange to blue indicates a high to low relative enrichment level.
- (J) Kaplan-Meier plots showing worse clinical outcome in bone metastatic samples with the higher proportion of osteoblasts-2 (account for all cells). Orange and blue lines indicate samples with high and low osteoblasts-2 proportion (median split), respectively. p value was calculated by log rank test.



Cell cycle group (Figure 4I), and a high proportion of osteoblasts-2 was associated with poor survival of bone metastatic patients (Figure 4J), implying the potential relationship between the differentiation of MSCs toward osteoblasts and the cancer cell proliferation under the bone metastatic microenvironment.

Myeloid subtypes exhibit distinct transcriptomic patterns in BMs

Considering the unique immune milieu provided by the bone marrow, it is imperative to systematically characterize the impact of bone microenvironment on the cancer cells that originate from diverse organs. As a key component of immune cells in the tumor microenvironment, myeloid cells play important roles in tumor progression and have been developed as anti-cancer therapies with an unprecedented number of clinical trials.^{89,90} Therefore, we focused on the myeloid cells in BMs and identified 5 lineages: myeloid-derived suppressor cells (MDSCs; 11,074), monocytes (3,675), macrophages (31,080), dendritic cells (DCs; 2,723), and neutrophils (247 cells). The calcium-binding proteins *S100A8* and *S100A9* were highly expressed in MDSCs, monocytes, and neutrophils. DCs and macrophages highly expressed major histocompatibility complex class II molecules (*HLA-DQA1*) and macrophage-derived apolipoprotein E (*APOE*), respectively (Figure 5A). Further clustering revealed 13 cell types of myeloid cells based on differentially expressed genes (Figures S5A and S5B). To assess the similarities of each lineage across cancer types, we computed correlations among the average transcriptomes of major lineages across various cancer types. As anticipated, major lineages from distinct cancer types were clustered together (Figure 5A), supporting that major myeloid lineage possessed analogous transcriptional profiles. Although macrophages and MDSCs constituted the largest proportion of myeloid cells (more than 50%) in the BM for most cancer types, their proportions showed drastic variation across different cancer types (Figure S5C). For example, infiltrating MDSCs constituted more than 25% of all myeloid cells in COAD and BRCA, while largely absent in MAM and NPC.

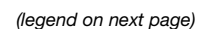
To understand the role of myeloid cells in the bone metastatic microenvironment, we next performed cell-cell interaction analysis and found that macrophages could interact with cancer

cells, though ligand-receptor pairs secreted phosphoprotein 1 (*SPP1*)-*CD44* and *SPP1*-integrin complex (Figure 5B). This potential interaction was validated by adjacent localization of the 2 types of cells in multiple BM cancers through immunofluorescence assay and subsequent quantification analysis (Figures 5C and 5D). The *SPP1* has been reported to be involved in tumor cell progression in multiple cancer types.^{91,92} Furthermore, the level of *SPP1* expressed by macrophages significantly associated with poor prognosis in patients with BMs (Figure 5E), indicating that the presence of *SPP1*⁺ macrophages may help the colonization of cancer cells in the BM.

As myeloid cells are highly plastic and can differentiate into diverse subtypes depending on the microenvironment,⁹⁰ it is intriguing to investigate the changes that myeloid subtypes undergo in the BM microenvironment. By integrating myeloid cells from normal bone tissues, diverse primary tumors, and other metastatic tumor tissues, we performed comparative analysis and found that MDSC-2 (highly expressed *C15orf48*, *TIMP1*, which was classified as M-MDSC), Macro-IFIT3, and osteoclasts were significantly enriched in BMs (Figures 5F and 5G). In addition, MDSC-2 and osteoclasts were enriched in BMs for most cancer types (Figure S5D). Comparison with paired normal bone marrow tissues and primary tumors also revealed the enrichment of osteoclasts in BMs (Figure S5E). As the principal effector cells of bone resorption, abnormally activated osteoclasts promote bone matrix degradation, thereby exacerbating tumor growth and dissemination.^{93,94} The enrichment of osteoclasts in BMs was further validated by tartrate-resistant acid phosphatase (TRAP) staining experiments (Figure 5H). Further trajectory analysis with CytoTRACE⁹⁵ revealed that BM-enriched subtype osteoclasts showed a more differentiated transcriptional state than the other myeloid cell types (Figure S5F), relative to other macrophage subsets and MDSC-1 (highly expressed *S100A12*, *LYZ*, and *ARG1*, which was classified as PMN-MDSC). In comparison with other myeloid cell subtypes, osteoclasts showed significantly upregulated expression of genes involved in osteoclast signaling and regulation of osteoclast development (Figures S5G and S5H). Moreover, with a higher abundance in the Cell cycle group (Figure 5I), osteoclast proportion was significantly associated with poor prognosis in

Figure 5. Subtypes of myeloid cells in pan-cancer bone metastases

- (A) Hierarchical clustering of major myeloid cell lineages across cancer types. Heatmap showing marker genes of major myeloid cell lineages. Color from red to white indicates a high to low expression.
- (B) Bubble plot showing interaction pairs between myeloid cells subtypes and cancer cells. Dot size indicates *p* value of interaction pairs, and color from red to white indicates a high to low average interaction intensity.
- (C) Representative mIHC images of bone metastases showing the spatial distribution of CD44⁺ tumor cells (CD44⁺ EpCAM⁺) around SPP1⁺ macrophages (SPP1⁺ CD68⁺). EpCAM (yellow), CD44 (magenta), CD68 (green), and SPP1 (red). Scale bars: 50 μ m (top) and 20 μ m (bottom).
- (D) Boxplot showing the percentage of CD44⁺ tumor cells at different distances to SPP1⁺ macrophages. Two-sided paired *t* test. *n* = 4.
- (E) Kaplan-Meier plots showing worse clinical outcome in bone metastatic samples with higher *SPP1* expression (median split) in macrophages. *p* value was calculated by log rank test.
- (F) Radar chart showing the infiltration proportions of myeloid cell subtypes in bone metastases, normal bone tissue, primary cancer, and other metastatic cancer. The radius was individually normalized for each cell subtype with the highest abundance of the cell subtype in different tissues.
- (G) Boxplot showing the percentages of osteoclasts in bone metastases (*n* = 53), normal bone tissues (*n* = 37), primary cancers (*n* = 156) and other metastatic cancers (*n* = 44). *p* values were calculated by Student's *t* tests.
- (H) Osteoclasts were identified by TRAP-positive staining in bone metastatic tissues from indicated original cancer types marked on top of each figure. The images in the dashed rectangle at the top panel were enlarged for visualization below. Scale bars: 2,000 μ m (top) and 500 μ m (bottom).
- (I) Heatmap showing the Ro/e ratio of various cell subtypes across the 3 cancer groups. The Ro/e ratio was calculated by one group versus the other 2 groups.
- (J) Kaplan-Meier plots showing worse clinical outcome in bone metastatic samples with higher proportion (median-split) of osteoclasts (in all macrophage cells). *p* value was calculated by log rank test.



bone metastatic patients (Figure 5J), consistent with the pro-tumor feature of osteoclasts in BMs.

Exhaustion of T lymphocytes in the BM microenvironment

Tumor-infiltrating T cells are crucial players in tumor microenvironment and have shaped fundamental clinical properties of patients.⁹⁶ Therefore, a comprehensive understanding of T cell repertoire in BMs is essential for advancing future therapies and improving patient prognosis. Utilizing the large-scale single cells from various BMs, we identified 34,288 CD4⁺ T cells, 56,790 CD8⁺ T cells, and 14,340 NKT cells across 12 cancer types (Figures S6A and S6B). Based on known functional marker genes, a number of 11 T cell subtypes were revealed in the BM (Figure S6C). CD4-Treg was characterized by the expression of canonical marker genes *FOXP3* and *CTLA4*. CD4 naive T cells (CD4-Tnaive) highly expressed *CCR7*, *SELL*, and *TCF7*; CD4 effector T cells (CD4-Teff) were characterized by high expression of ISG-associated genes, CD4-Tctl (cytotoxic T lymphocytes) displayed high expression of cytotoxic effector genes (*GZMA*, *GZMB* and *PRF1*),^{97–99} and exhausted CD4⁺ T cells (CD4-Tex) were featured by expression of exhaustion genes (*PDCD1*, *CXCL13*, and *TOX*).^{100,101} Among CD8⁺ T cells, 2 effector T cells (CD8-Teff1 and CD8-Teff2) were characterized by expression of effector genes *GZMA*, *PRF1* and *KLRG1*,¹⁰² with CD8-Teff2 having a relatively high expression of interferon response gene *IFIT3*. The high expression of IFN genes in CD8 T effector cells has been reported in several studies.^{23,103} Two effector memory-like T cells (CD8-Tem1 and CD8-Tem2) were characterized by expression of *GZMK* and *IL7R*.^{99,104,105} (Figures 6A and S6C). One exhausted CD8⁺ T cluster (CD8-Tex) was identified by high expression of exhaustion markers (*PDCD1*, *HAVCR2*, *CXCL13*, *TIGIT*, and *LAG3*)¹⁰⁶ (Figures S6C and S6D).

Hierarchical clustering of gene expression profiles for various T cell subtypes across different cancer types revealed that most of the same subtypes from different cancer types were

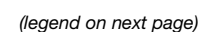
clustered together (Figure 6A), indicating that T cell subtypes shared similar transcriptomic profiles across cancer types. The proportion of T cell subtypes varied among different cancer types, with more infiltration of CD8-Tex in RCC and COAD, whereas CD8-Teff more abundant in THCA and myofibroblastoma (Figure S6E). Furthermore, by integrating T cells from diverse primary cancer types, normal bone marrows, and other metastatic tissues, we found that CD4-Teff, CD4-Treg, CD8-Teff2, and CD8-Tex infiltrated more in BMs than other tissues (Figure 6B). Further comparison in paired tissues consistently showed the enrichment of these cell subtypes in BMs compared to the derived primary tumors or adjacent normal bone tissues (Figure S6F). This phenomenon may contribute to the aggressive progression and therapeutic resistance characteristic of bone metastatic tumors.

Heterogeneity of CD8⁺ T subtypes is closely associated with response to checkpoint blockade in tumor.^{107–109} Thus, we then performed cell trajectory analysis by RNA velocity¹¹⁰ to understand functional transitions of CD8⁺ T cell subtypes. The results showed a differentiation path from CD8-Teff or CD8-Tem to CD8-Tex cells, with the CD8-Tex as the terminal of the trajectory (Figure 6C). Bone metastatic tumors showed significantly higher abundance of CD8-Tex than normal bone tissues, primary tumors, or other metastatic tumors (Figure 6D). In addition, CD8-Tex showed higher abundance in BMs than primary tumors for a majority of cancer types (Figure S6G). Across the 3 cancer groups defined by transcriptional programs, CD8-Tex subtype was more abundant in the Cell cycle and Migrate groups (Figures 6E and S6H). Consistently, BM patients with higher proportion of CD8-Tex cells were associated with poor prognosis (Figure 6F).

We further investigated transcription factors within the BM microenvironment that may influence the differentiation and function of CD8 T cell subsets. The results revealed higher transcriptional activity of ETS1 and ATF2 in CD8-Tex (Figure 6G). Among these, ETS1 expression was significantly elevated in CD8-Tex cells from bone metastatic lesions compared to those

Figure 6. Subtypes of T cells in pan-cancer bone metastases

- Hierarchical clustering of major T cell lineages across cancer types. Heatmap showing marker genes of major T cell lineages. Dot size indicates fraction of cells with expression of indicated genes, and color from red to white indicates a high to low normalized expression.
- Radar chart showing the infiltration proportions of T cell subtypes in bone metastases, normal bone tissues, primary, and other metastatic tumor tissues. The radius was individually normalized for each cell subtype with the highest abundance of the cell subtype in different tissues.
- (Upper) RNA velocities overlaid on UMAP of CD8⁺ T cells subtypes. Arrows show the RNA velocity field. Cells are colored by CD8⁺ T cell subtypes labeled on plot. (Lower) Pseudotime overlaid on the UMAP of CD8⁺ T cells subtypes. Color from purple to red indicates a 0 to 1 pseudotime.
- Boxplot showing the percentages of CD8-Tex in bone metastases ($n = 53$), normal bone tissues ($n = 37$), primary cancers ($n = 182$), and other metastatic cancers ($n = 44$). p values were calculated by Student's t tests.
- Lollipop chart illustrates the Ro/e ratio of CD8-Tex across the 3 cancer groups. The Ro/e ratio was calculated by one group versus the other two groups.
- Kaplan-Meier plots showing worse clinical outcome in bone metastatic samples with higher proportion of CD8-Tex (median-split). p value was calculated by log rank test.
- Heatmap displaying the transcription factor activity of CD8T subsets. Color from orange to blue indicates a high to low activity.
- Heatmap showing the transcription factor activity across bone metastases, normal bone tissues, primary cancers, and other metastatic cancers. Color from yellow to black indicates a high to low activity.
- Transcriptional expression of target genes by ETS1.
- Heatmap showing the normalized expression of immune checkpoint genes along the trajectory path (pseudotime) of CD8 T cells. Color from orange to blue indicates a high to low normalized expression.
- (Left) Heatmap showing the mean expression levels of immune checkpoint genes across bone metastases, normal bone tissues, primary cancers, and other metastatic cancers. (Right) Heatmap showing the mean expression levels of immune checkpoint genes in paired bone metastases and primary tumor tissues from the same patients. Color from yellow to black indicates a high to low normalized expression.
- Kaplan-Meier plots showing the relationship between clinical outcome in bone metastatic patients and expression level of *PDCD1* and *TIGIT* (median-split) in T cells. p values were calculated by log rank tests.



in normal bone, primary tumors, and other metastatic cancers (Figure 6H). ETS1 has been previously reported to be critical for the terminal exhaustion of CD8-Tex cells.¹¹¹ We further examined the expression of ETS1 downstream target genes in CD8-Tex cells from BMs (Figure 6I) and found that genes involved in T cell responses to environmental stimuli and cellular development, such as *CD8A* and *LCK*, were upregulated under the targeting by ETS1 (Figure S6I).

Exhausted CD8⁺ T cells were reported to be associated with resistance to immunotherapy.^{105,112,113} We next examined the expression pattern of immune checkpoint genes in BMs and found that most of these genes showed increasing expression along the CD8⁺ T cell trajectory path (Figure 6J). In addition, *PDCD1*, *TIGIT*, and *HAVCR2* showed the highest expression level in BMs compared to normal bone, primary tumor, or other metastatic tumors (Figure 6K). The expression of these genes showed consistently higher level in BMs than their paired primary tumors (Figure 6K). Furthermore, higher expressions of *PDCD1* and *TIGIT* were significantly associated with poorer survival of BM patients (Figure 6L). Together, these results suggested the potential role of *TIGIT* and *PDCD1* in T cell exhaustion and poor survival of BMs.

TIGIT inhibitors reshaped the composition of T cell subtypes in BMs

Current treatment strategies including target therapy, radiotherapy, and chemotherapy all showed no improvement in the survival probability of BM patients (Figure S7A), raising the urgent need for new treatment options. Therefore, given the potential role of *TIGIT* and *PDCD1* in the BM, we next explored their therapeutic potential using mouse models. Specifically, we constructed a bone metastatic mouse model by intra-iliac artery injection according to the protocol reported before.¹³ At 7, 9, 11, 13, and 15 days after the establishment of the mouse model, we treated these mice with antibodies targeting Pd-1 or Tigit or both by intraperitoneal injection, with the control group treated with IgG antibody (Figure S7B). We first examined the tumor mass after 5 days of the last treatment (20 days after the model establishment) using *in vivo* imaging system (IVIS) and found

that both anti-Pd-1 and anti-Tigit treatment reduced the tumor burden, while the dual treatment group showed the highest reduction level (Figures 7A and S7C). In addition, the micro-computed tomography (CT) results showed that combined treatment effectively reduced bone destruction by the highest level (Figures 7B and S7D). Moreover, the results of TRAP staining showed combined anti-Pd-1 and Tigit therapy significantly inhibited the activation of osteoclasts (Figure 7C). To further evaluate the effects of single or dual treatment, we next performed immunohistochemistry (IHC) staining to examine the changes in tumor cell proliferation- (Ki67) and apoptosis- (cleaved caspase-3/CC3 and cleaved caspase-9/CC9) associated protein markers. Our quantitative analysis revealed a significant reduction in Ki67⁺ tumor cells in the combined therapy group compared to those treated with monotherapy or control. Concurrently, we observed a marked increase in the expression of CC3 and CC9, confirming that the combined therapy effectively induced apoptosis in tumor cells (Figures 7D and S8).

Furthermore, we evaluated the cytotoxic potential of CD8⁺ T cells within the tumor microenvironment by assessing the expression of key cytotoxic effector molecules, including granzyme B (GZMB), perforin (PF), tumor necrosis factor (TNF)- α , and IFN- γ . Strikingly, the combined therapy group demonstrated a substantial upregulation of these cytotoxic mediators compared to monotherapy and control groups (Figures 7D and S8), suggesting a potent activation of CD8⁺ T cell-mediated antitumor immunity. To elucidate the effects of the treatment on immune profiles in the mouse model, we next performed high-dimensional mass cytometry using cytometry by time-of-flight (CyTOF) analysis for the treated mice at 3 days of the last treatment (17 days after the model establishment). Unsupervised clustering of proteomic profiles of Cd45⁺ cells revealed 8 immune types with classical marker genes (Figures S7E and S7F). The abundance of myeloid cell types was increased in mice treated with anti-Pd-1 or dual antibodies (Figure S7G). Subsequent analysis of the myeloid compartment revealed a greater enrichment of macrophages following anti-Pd-1 or combination therapy (Figure S7G). Examination of macrophage subpopulations further demonstrated an increase in the Macro-Mertk subgroup, characterized by

Figure 7. TIGIT and PD-1 combination treatment of bone metastases in mice

(A) (Left) Representative images from IVIS showing tumor burdens (radiance [p/s/cm²/sr]) in intra-iliac artery injection mouse models of the control and each treatment group at the endpoint of the experiment (20 days after the successful establishment of bone metastatic mouse model). Images from other mice were shown in Figure S7C. (Right) Quantitative analysis of tumor burden in all mice across the 4 experimental groups (*n* = 6). All results are presented as mean $\pm$ SD. Each data point in bar plots represents one subject. Statistical significance was determined by one-way ANOVA.

(B) (Left) Representative micro-CT images showing the bone intensity of femora/tibiae from the control and treated mice. Images from other mice were shown in Figure S7D. Scale bar, 2 mm. (Right) Quantitative analysis of bone intensity (quantified by micro-CT bone volume/total volume %) in all mice across the 4 groups (*n* = 3). All results are presented as mean $\pm$ SD. Each data point in bar plots represents one subject. Statistical significance was determined by one-way ANOVA.

(C) (Left) Representative images of TRAP staining results showing the abundance of osteoclasts in the control and treatment groups. Scale bar, 100 μ m. (Right) Quantitative analysis of osteoclasts abundance (TRAP staining) across the 4 groups (*n* = 6). All results are presented as mean $\pm$ SD. Each data point in bar plots represents one subject. Statistical significance was determined by one-way ANOVA.

(D) (Top) Representative images of Ki67 (a marker of cell proliferation), CC3 (cleaved caspase-3, a marker of apoptosis), and CC9 (cleaved caspase-9, another marker of apoptosis) staining (left) as well as cytotoxic CD8⁺ T cell infiltration and effector molecule expression (granzyme B [GZMB], PF, TNF- α , and IFN- γ) in bone metastatic tissues of the model mice (*n* = 6). Scale bar, 25 μ m. (Bottom) Quantitative analysis of the indicated markers from immunostaining results across the 4 groups. All results are presented as mean $\pm$ SD. Each data point in bar plots represents one subject. Statistical significance was determined by one-way ANOVA.

(E) UMAP visualizations of T cell subtypes detected using mass spectrometry. Cells are colored by cell types labeled on legend.

(F) UMAP visualizations of protein expression for markers of various T cell subtypes. Color from pink to gray indicates a high to low normalized expression.

(G) Heatmap showing the average percentage of indicated CD8 T cell subtypes in each experimental group from CyTOF analysis. **p* < 0.05 by Bayesian model from the scCODA method.¹¹⁴

phagocytic gene signatures, while the Macro-Sirpa subgroup, associated with dysfunctional signatures, was reduced after dual antibody treatment (Figure S7H). Notably, prior research identified Macro-Sirpa as a contributor to the establishment of an immunosuppressive microenvironment in BMs,³⁸ indicating that dual treatment may enhance tumor control by inhibiting the infiltration of this heterogeneous macrophage subpopulation. Meanwhile, more in-depth clustering of T cells revealed 6 subtypes of Cd4⁺ T cells, 5 subtypes of Cd8⁺ T cells, 1 double positive T cells (DPT), and 1 double negative T cells (DNT) based on the heterogeneity of protein expression profiles (Figures 7E and 7F). The composition of different T cell subtypes also changed after the treatment (Figure S7I). For example, the abundance of Cd8-Tex was significantly reduced in the group treated with anti-Pd-1 antibody and dual antibodies. In addition, the proportion of CD8 T effector cells was increased upon the dual treatment of both Pd-1 and Tigit antibodies (Figure 7G). Collectively, our comprehensive analysis has depicted the immunological impact of different treatments on BM tumor. The combined therapy not only effectively suppresses tumor cell proliferation and promotes apoptosis but also significantly enhances the cytotoxic activity of CD8⁺ T cells, thereby providing a more robust antitumor immune response. These findings underscore the potential of the combined therapy as a promising strategy for the treatment of BM tumors and warrant further investigation in clinical settings.

DISCUSSION

In this study, we systematically characterized the composition and heterogeneity of various cell types in the BMs that originate from various primary tumor sites, primarily utilizing samples obtained from spinal metastatic sites. The unprecedented scale of the datasets from both BMs and primary cancers together with healthy bone marrows enabled us to understand cancer cell properties and tumor microenvironment characteristics in the bone metastatic niche. Integrative analysis revealed *SELE*-positive EC, osteoblast, osteoclast, and exhausted CD8 T cell subtypes associated with the cancer cell proliferation in BMs. The increased expression of immune checkpoint genes in BMs triggered us to explore the potential of a combined immune therapy in bone metastatic mouse models. The comprehensive catalog of functional cell types and cellular states generated in this study also provides a resource to the research community for future exploration of BMs. An interactive website is available through <http://www.sunlab.fun:8888/scBoneAtlas/>.

Based on the gene expression profiles of tumor cells, we identified 15 consensus tumor programs from bone metastatic cancer cells derived from diverse cancer types. This result is in line with the notion that distant metastases are composed of genetically homogeneous cell populations.^{115,116} The higher level of Cell cycle programs in BMs compared with primary tumors and the worst survival probability of patients in the Cell cycle group indicated the advanced progression of cancer cells in the bone metastatic microenvironment. Moreover, we identified molecular features upregulated in the bone metastatic niche, especially the activation of the MYC signaling pathway and elevated expression levels of *NME2*. Higher level of both features was associated with poor prognosis in BMs. MYC has been well

established to play an important role in tumor metastasis.^{117,118}

As a transcriptional regulator of MYC, *NME2* has been shown to bind to the nuclease-hypersensitive element (NHEIII) in the promoter region of MYC interacting with G-quadruplex structure and increase MYC expression.^{119,120} The *NME2* has been shown to be involved in tumorigenesis and treatment response across different cancer types.^{121–123} Our results here nominate MYC signaling pathway and *NME2* as potential therapeutic targets for bone metastatic patients with high expression profiles of these molecular markers especially in the Cell cycle group.

For stromal cells, we found the spatial enrichment of *SELE* within vein cells and vein-like capillaries in BMs, suggesting a potential vascular specialization that may extend beyond its traditional role in leukocyte trafficking.¹²⁴ Notably, *SELE* has recently been identified as a functional niche molecule that interacts with tumor cells to induce a noncanonical MET and activate Wnt signaling, thereby promoting tumor cell colonization within the bone microenvironment.⁷⁶ Consistent with these findings, our data indicate that *SELE*⁺ vein cells and vein-like capillaries in the bone vascular niche may form an adhesive and signaling interface that contributes to hematogenous tumor dissemination and subsequent adaptation of tumor cells within the bone microenvironment.

The interaction between mesenchymal stromal cells and cancer cells is well recognized in regulating the carcinogenesis process at different stages.¹²⁵ The bonding of type I collagen on the mesenchymal stromal cells and integrin on the cancer cells has been reported to promote tumor growth and metastasis in colorectal cancer and ovarian cancer.^{126,127} Here, we demonstrated that *COL1A1-ITGB1* binding might be involved in the adhesion of tumor cells through mesenchymal stromal cells in the BM microenvironment. The osteogenic signaling has been known to participate in the bone remodeling process in BMs of various tumor diseases, and antagonists of osteogenic signaling are pharmacological targets in the treatment of osteoporosis.^{128–130} In the present study, we have found a subtype of osteoblasts with high expression of *ENTHD1* and *SLC7A11*. Previous studies have shown that *SLC7A11* could import cystine for glutathione (GSH) synthesis, and high level of GSH can cause abnormal osteoblast differentiation, which plays a key role in tumor BM.¹³¹ The abnormally differentiated osteoblasts would cause the increased concentration of protons to influence the progression or symptoms of BM in many different ways, which protect cancer cells from oxidative stress and ferroptosis to enhance cancer cell motility and aggressiveness in the bone microenvironment.¹³² Besides, tumor-derived *SLC7A11* activity may alter osteoblast-osteoclast coupling, contributing to osteolytic or osteosclerotic lesions.¹³³ *ENTHD1* belongs to the Epsin protein family, which mediated endocytosis and could promote cancer cell invasion.¹³⁴ The regulation is closely related with their characteristic Epsin N-terminal homology (ENTH) domain, which is necessary for cell migration and invasion. All the Epsin endocytic motifs reside in the C-terminal part of the molecule, and their existence plays a critical role in the interaction between Epsin protein family and other proteins. This osteoblast subtype was also characterized by activated hypoxic activity and associated with poor survival of bone metastatic patients, pointing a potential therapeutic potential for BM treatment.

As essential immune cells in the tumor microenvironment, macrophages that are differentiated from MDSCs have been reported to show immunosuppressive characteristics.¹³⁵ Here, we revealed the heterogeneity of various myeloid cell types in BMs and found the strong interaction between SPP1⁺ macrophages and cancer cells through SPP1-CD44 or SPP1-integrin. SPP1⁺ tumor-associated macrophages were reported to be associated with angiogenesis, immunosuppression, and shorter survival in patients with multiple cancers,^{136,137} supporting our results on the poor prognosis associated with SPP1⁺ macrophages. In addition, we found that osteoclasts turned out to be the terminal state of the macrophage differentiation in the BM, in line with previous reports in lung, colorectal, and ovarian cancers.²¹ Furthermore, the osteoclast subtype was specifically enriched in bone metastatic tumors in comparison with normal bone tissues or primary or other metastatic tumors for most cancer types, indicating its unique role in the bone metastatic niche. Together with its high abundance in the Cell cycle group and association with poor prognosis, this subtype of macrophages is worth to be further investigated for its specific role in tumor progression and as therapeutic target in BM.

Accumulating evidence indicates that BMs exhibit reduced sensitivity or resistance to immune checkpoint inhibitors (ICIs).^{138,139} Current clinical data suggest that anti-TIGIT monotherapy demonstrates limited antitumor activity, whereas combination therapies targeting both TIGIT and PD-1/PD-L1 have enhanced efficacy across various tumor types.⁹³ In the present study, we found that the expression of immune checkpoint genes increased along the differentiation trajectory and explored the therapeutic potential of antibodies targeting PD-1 and TIGIT in the bone metastatic mouse model. Our results suggested that combinatorial immune therapy by targeting both PD-1 and TIGIT could efficiently reshape the immune microenvironment and prevent the progression of tumor cells in BM mouse models, supporting the necessity for consideration of combined immunotherapy targeting in the treatment of BM patients. Furthermore, our study has identified several potential therapeutic targets that could expand the treatment options for BMs. These targets include *NME2* and *MYC* within tumor cells, as well as *SELE*⁺ EC5 and *MSC-LRRC15*⁺ stromal subsets within the bone metastatic microenvironment. Rather than functioning as isolated entities, these molecules and cell populations serve as integrative nodes that link tumor-intrinsic signaling with the remodeling of microenvironment. As shown in previous studies, *SELE* is located in endothelium and activated by cytokines, which has been verified to help metastatic tumor cells adhere to ECs in the bone marrow.^{76,140} Besides, *LRRC15* has been found to be correlated with the expression of *TWIST1*, which contributes to the stemness of MSCs.¹⁴¹ Especially, the high level of *LRRC15* was verified in cancer-associated fibroblasts,¹⁴² which implies the important role in tumor immune microenvironment.¹⁴³ Targeting these nodes has the potential not only to complement current ICIs but also, when further integrated with conventional modalities such as chemotherapy, targeted therapy, or radiotherapy, to produce synergistic effects that remodel the bone microenvironment and overcome therapeutic resistance. Future research should focus on spatially resolved and mechanistic investigations of these cellular interactions. The integration of spatial multi-omics, lineage tracing, and functional perturbation models

will be essential to elucidate the causal hierarchy among endothelial, stromal, and immune compartments. Furthermore, translating these findings into early-phase clinical exploration could facilitate the identification of actionable molecular vulnerabilities and the validation of combination strategies specifically tailored to the bone metastatic niche.

Limitations of the study

Despite that our single-cell transcriptomic dataset surpasses previous attempts at comprehensively analyzing bone metastatic tumors or other distant metastases, the current study is still limited by the small number of matched primary tumors or other metastatic tumors given the challenges in sample collection. In addition, megakaryocytes, basophils, and eosinophils were not detected in our datasets, possibly related to the sample collection and sequencing method, which could miss cells with large diameters. Moreover, the prognosis of BM patients might be confounded by treatment during the removal of their primary tumors long before the occurrence of BMs. Beyond these technical and sampling limitations, our validation analyses were primarily correlative. Consequently, future research should integrate functional experiments to transcend mere correlation and clarify the mechanistic foundations of our findings. Furthermore, it is essential for future studies to infer clinical outcomes and the impact of different therapies with larger cohort of patients with more comprehensive clinical information.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for the resources and reagents may be directed to and will be fulfilled by the lead contact, Yidi Sun (ydsun@ion.ac.cn).

Materials availability

All materials used for scRNA-seq, immunostaining, and *in vivo* experiments are commercially available.

Data and code availability

An interactive website for querying the processed data is accessible via <http://www.sunlab.fun:8888/scBoneAtlas/>. All processed and raw data are accessible through the National Omics Data Encyclopedia (accession code: OEP005136, <https://www.biosino.org/node/project/detail/OEP005136>). Data download guidelines can be found in Methods S1. All data were analyzed with standard programs and packages, as detailed in the STAR Methods. Custom code of data analysis and visualization is available at <https://github.com/FenMaMuffin/scBoneAtlas>. Additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y. Shao, W.Y., and Y. Sun; resources, S.W., D.W., L.X., Q. Ye, W.H., Z.S., Z.W., W.C., M.F., M.C., Y.J., Y.Z., B.G., S.S., and W.Y.; methodology, S.W., F.M., D.W., Q. Yu, H.Z., and Y. Sun; investigation, S.W., F.M., and D.W.; formal analysis, S.W., F.M., and Y.T.; validation, S.W., F.M., and Y. Sun; data curation, S.W., F.M., W.Y., and Y. Sun; visualization, S.W. and F.M.; writing – original draft, S.W., F.M., Q. Yu, H.Z., S.Z., and Y. Sun; writing – review & editing, S.W., F.M., Q. Yu, H.Z., S.Z., Y. Shao, and Y. Sun; supervision, S.W., Y. Shao, W.Y., and Y. Sun.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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REFERENCES

1. Coleman, R.E. (2001). Metastatic bone disease: clinical features, pathophysiology and treatment strategies. *Cancer Treat Rev.* 27, 165–176. <https://doi.org/10.1053/ctrv.2000.0210>.
2. Mundy, G.R. (2002). Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat. Rev. Cancer* 2, 584–593. <https://doi.org/10.1038/nrc867>.
3. Portales, F., Thézenas, S., Samalin, E., Assenat, E., Mazard, T., and Ychou, M. (2015). Bone metastases in gastrointestinal cancer. *Clin. Exp. Metastasis* 32, 7–14. <https://doi.org/10.1007/s10585-014-9686-x>.
4. Roodman, G.D. (2004). Mechanisms of bone metastasis. *N. Engl. J. Med.* 350, 1655–1664. <https://doi.org/10.1056/NEJMra030831>.
5. Coleman, R., Body, J.J., Aapro, M., Hadji, P., and Herrstedt, J.; ESMO Guidelines Working Group (2014). Bone health in cancer patients: ESMO Clinical Practice Guidelines. *Ann. Oncol.* 25, iii124–iii137. <https://doi.org/10.1093/annonc/mdu103>.
6. von Moos, R., Costa, L., Gonzalez-Suarez, E., Terpos, E., Niepel, D., and Body, J.J. (2019). Management of bone health in solid tumours: From bisphosphonates to a monoclonal antibody. *Cancer Treat Rev.* 76, 57–67. <https://doi.org/10.1016/j.ctrv.2019.05.003>.
7. Coleman, R.E., Croucher, P.I., Padhani, A.R., Clézardin, P., Chow, E., Fallon, M., Guise, T., Colangeli, S., Capanna, R., and Costa, L. (2020). Bone metastases. *Nat. Rev. Dis. Primers* 6, 83. <https://doi.org/10.1038/s41572-020-00216-3>.
8. Wewel, J.T., and O'Toole, J.E. (2020). Epidemiology of spinal cord and column tumors. *Neuro-Oncol. Pract.* 7, i5–i9. <https://doi.org/10.1093/nop/npaa046>.
9. Grosinger, A.J., and Alcorn, S.R. (2024). An Update on the Management of Bone Metastases. *Curr. Oncol. Rep.* 26, 400–408. <https://doi.org/10.1007/s11912-024-01515-8>.
10. Carpenter, K., Decater, T., Iwanaga, J., Maulucci, C.M., Bui, C.J., Dumont, A.S., and Tubbs, R.S. (2021). Revisiting the Vertebral Venous Plexus-A Comprehensive Review of the Literature. *World Neurosurg.* 145, 381–395. <https://doi.org/10.1016/j.wneu.2020.10.004>.
11. Jacob, L., Boisserand, L.S.B., Geraldo, L.H.M., de Brito Neto, J., Mathivet, T., Antila, S., Barka, B., Xu, Y., Thomas, J.M., Pestel, J., et al. (2019). Anatomy and function of the vertebral column lymphatic network in mice. *Nat. Commun.* 10, 4594. <https://doi.org/10.1038/s41467-019-12568-w>.
12. Baldassarri, I., Tavakol, D.N., Graney, P.L., Chramiec, A.G., Hibshoosh, H., and Vunjak-Novakovic, G. (2024). An engineered model of metastatic colonization of human bone marrow reveals breast cancer cell re-modeling of the hematopoietic niche. *Proc. Natl. Acad. Sci. USA* 121, e2405257121. <https://doi.org/10.1073/pnas.2405257121>.
13. Zhang, W., Bado, I.L., Hu, J., Wan, Y.W., Wu, L., Wang, H., Gao, Y., Jeong, H.H., Xu, Z., Hao, X., et al. (2021). The bone microenvironment invigorates metastatic seeds for further dissemination. *Cell* 184, 2471–2486.e20. <https://doi.org/10.1016/j.cell.2021.03.011>.
14. Prasad, D., and Schiff, D. (2005). Malignant spinal-cord compression. *Lancet Oncol.* 6, 15–24. [https://doi.org/10.1016/S1470-2045\(04\)01709-7](https://doi.org/10.1016/S1470-2045(04)01709-7).
15. Liu, F., Ding, Y., Xu, Z., Hao, X., Pan, T., Miles, G., Wang, S., Wu, Y.H., Liu, J., Bado, I.L., et al. (2025). Single-cell profiling of bone metastasis ecosystems from multiple cancer types reveals convergent and divergent mechanisms of bone colonization. *Cell Genom.* 5, 100888. <https://doi.org/10.1016/j.xgen.2025.100888>.
16. Clarke, B. (2008). Normal bone anatomy and physiology. *Clin. J. Am. Soc. Nephrol.* 3, S131–S139. <https://doi.org/10.2215/CJN.04151206>.
17. Satcher, R.L., and Zhang, X.H.F. (2022). Evolving cancer-niche interactions and therapeutic targets during bone metastasis. *Nat. Rev. Cancer* 22, 85–101. <https://doi.org/10.1038/s41568-021-00406-5>.
18. Bado, I.L., Zhang, W., Hu, J., Xu, Z., Wang, H., Sarkar, P., Li, L., Wan, Y.W., Liu, J., Wu, W., et al. (2021). The bone microenvironment increases

- phenotypic plasticity of ER(+) breast cancer cells. *Dev. Cell* 56, 1100–1117.e9. <https://doi.org/10.1016/j.devcel.2021.03.008>.
19. Mishra, A., Shiozawa, Y., Pienta, K.J., and Taichman, R.S. (2011). Homing of cancer cells to the bone. *Cancer Microenviron.* 4, 221–235. <https://doi.org/10.1007/s12307-011-0083-6>.
20. Ottewill, P.D., O'Donnell, L., and Holen, I. (2015). Molecular alterations that drive breast cancer metastasis to bone. *BoneKey Rep.* 4, 643. <https://doi.org/10.1038/bonekey.2015.10>.
21. Qian, J., Olbrecht, S., Boeckx, B., Vos, H., Laoui, D., Etlioglu, E., Wauters, E., Pomella, V., Verbandt, S., Busschaert, P., et al. (2020). A pan-cancer blueprint of the heterogeneous tumor microenvironment revealed by single-cell profiling. *Cell Res.* 30, 745–762. <https://doi.org/10.1038/s41422-020-0355-0>.
22. Cheng, S., Li, Z., Gao, R., Xing, B., Gao, Y., Yang, Y., Qin, S., Zhang, L., Ouyang, H., Du, P., et al. (2021). A pan-cancer single-cell transcriptional atlas of tumor infiltrating myeloid cells. *Cell* 184, 792–809.e23. <https://doi.org/10.1016/j.cell.2021.01.010>.
23. Zheng, L., Qin, S., Si, W., Wang, A., Xing, B., Gao, R., Ren, X., Wang, L., Wu, X., Zhang, J., et al. (2021). Pan-cancer single-cell landscape of tumor-infiltrating T cells. *Science* 374, abe6474. <https://doi.org/10.1126/science.abe6474>.
24. Tang, F., Li, J., Qi, L., Liu, D., Bo, Y., Qin, S., Miao, Y., Yu, K., Hou, W., Li, J., et al. (2023). A pan-cancer single-cell panorama of human natural killer cells. *Cell* 186, 4235–4251.e20. <https://doi.org/10.1016/j.cell.2023.07.034>.
25. Zhang, X., Xiao, K., Wen, Y., Wu, F., Gao, G., Chen, L., and Zhou, C. (2024). Multi-omics with dynamic network biomarker algorithm prefigures organ-specific metastasis of lung adenocarcinoma. *Nat. Commun.* 15, 9855. <https://doi.org/10.1038/s41467-024-53849-3>.
26. Kfoury, Y., Baryawno, N., Severe, N., Mei, S., Gustafsson, K., Hirz, T., Brouse, T., Scadden, E.W., Iolkina, A.A., Kokkalis, K., et al. (2021). Human prostate cancer bone metastases have an actionable immunosuppressive microenvironment. *Cancer Cell* 39, 1464–1478.e8. <https://doi.org/10.1016/j.ccell.2021.09.005>.
27. Mei, S., Alchahin, A.M., Tsea, I., Kfoury, Y., Hirz, T., Jeffries, N.E., Zhao, T., Xu, Y., Zhang, H., Sarkar, H., et al. (2024). Single-cell analysis of immune and stroma cell remodeling in clear cell renal cell carcinoma primary tumors and bone metastatic lesions. *Genome Med.* 16, 1. <https://doi.org/10.1186/s13073-023-01272-6>.
28. Azizi, E., Carr, A.J., Plitas, G., Cornish, A.E., Konopacki, C., Prabhakaran, S., Nainys, J., Wu, K., Kisilevov, V., Setty, M., et al. (2018). Single-Cell Map of Diverse Immune Phenotypes in the Breast Tumor Microenvironment. *Cell* 174, 1293–1308.e36. <https://doi.org/10.1016/j.cell.2018.05.060>.
29. Pelka, K., Hofree, M., Chen, J.H., Sarkizova, S., Pirl, J.D., Jorgji, V., Bejnood, A., Dionne, D., Ge, W.H., Xu, K.H., et al. (2021). Spatially organized multicellular immune hubs in human colorectal cancer. *Cell* 184, 4734–4752.e20. <https://doi.org/10.1016/j.cell.2021.08.003>.
30. Che, L.H., Liu, J.W., Huo, J.P., Luo, R., Xu, R.M., He, C., Li, Y.Q., Zhou, A.J., Huang, P., Chen, Y.Y., et al. (2021). A single-cell atlas of liver metastases of colorectal cancer reveals reprogramming of the tumor microenvironment in response to preoperative chemotherapy. *Cell Discov.* 7, 80. <https://doi.org/10.1038/s41421-021-00312-y>.
31. Liu, Y., Zhang, Q., Xing, B., Luo, N., Gao, R., Yu, K., Hu, X., Bu, Z., Peng, J., Ren, X., and Zhang, Z. (2022). Immune phenotypic linkage between colorectal cancer and liver metastasis. *Cancer Cell* 40, 424–437.e5. <https://doi.org/10.1016/j.ccell.2022.02.013>.
32. Lenos, K.J., Bach, S., Ferreira Moreno, L., Ten Hoorn, S., Sluiter, N.R., Bootsma, S., Vieira Braga, F.A., Nijman, L.E., van den Bosch, T., Miedema, D.M., et al. (2022). Molecular characterization of colorectal cancer related peritoneal metastatic disease. *Nat. Commun.* 13, 4443. <https://doi.org/10.1038/s41467-022-32198-z>.
33. Lu, Y., Yang, A., Quan, C., Pan, Y., Zhang, H., Li, Y., Gao, C., Lu, H., Wang, X., Cao, P., et al. (2022). A single-cell atlas of the multicellular ecosystem of primary and metastatic hepatocellular carcinoma. *Nat. Commun.* 13, 4594. <https://doi.org/10.1038/s41467-022-32283-3>.
34. Kim, N., Kim, H.K., Lee, K., Hong, Y., Cho, J.H., Choi, J.W., Lee, J.I., Suh, Y.L., Ku, B.M., Eum, H.H., et al. (2020). Single-cell RNA sequencing demonstrates the molecular and cellular reprogramming of metastatic lung adenocarcinoma. *Nat. Commun.* 11, 2285. <https://doi.org/10.1038/s41467-020-16164-1>.
35. Liu, Y., He, S., Wang, X.L., Peng, W., Chen, Q.Y., Chi, D.M., Chen, J.R., Han, B.W., Lin, G.W., Li, Y.Q., et al. (2021). Tumour heterogeneity and intercellular networks of nasopharyngeal carcinoma at single cell resolution. *Nat. Commun.* 12, 741. <https://doi.org/10.1038/s41467-021-21043-4>.
36. Chen, S., Zhu, G., Yang, Y., Wang, F., Xiao, Y.T., Zhang, N., Bian, X., Zhu, Y., Yu, Y., Liu, F., et al. (2021). Single-cell analysis reveals transcriptomic remodellings in distinct cell types that contribute to human prostate cancer progression. *Nat. Cell Biol.* 23, 87–98. <https://doi.org/10.1038/s41556-020-00613-6>.
37. Kang, B., Camps, J., Fan, B., Jiang, H., Ibrahim, M.M., Hu, X., Qin, S., Kirchhoff, D., Chiang, D.Y., Wang, S., et al. (2022). Parallel single-cell and bulk transcriptome analyses reveal key features of the gastric tumor microenvironment. *Genome Biol.* 23, 265. <https://doi.org/10.1186/s13059-022-02828-2>.
38. Ma, F., Wang, S., Xu, L., Huang, W., Shi, G., Sun, Z., Cai, W., Wu, Z., Huang, Y., Meng, J., et al. (2024). Single-cell profiling of the microenvironment in human bone metastatic renal cell carcinoma. *Commun. Biol.* 7, 91. <https://doi.org/10.1038/s42003-024-05772-y>.
39. Oetjen, K.A., Lindblad, K.E., Goswami, M., Gui, G., Dagur, P.K., Lai, C., Dillon, L.W., McCoy, J.P., and Hourigan, C.S. (2018). Human bone marrow assessment by single-cell RNA sequencing, mass cytometry, and flow cytometry. *JCI Insight* 3, e124928. <https://doi.org/10.1172/jci.insight.124928>.
40. Bandyopadhyay, S., Duffy, M.P., Ahn, K.J., Sussman, J.H., Pang, M., Smith, D., Duncan, G., Zhang, I., Huang, J., Lin, Y., et al. (2024). Mapping the cellular biogeography of human bone marrow niches using single-cell transcriptomics and proteomic imaging. *Cell* 187, 3120–3140.e29. <https://doi.org/10.1016/j.cell.2024.04.013>.
41. Gonzalez, H., Mei, W., Robles, I., Hagerling, C., Allen, B.M., Hauge Okholm, T.L., Nanjaraj, A., Verbeek, T., Kalavachera, S., van Gogh, M., et al. (2022). Cellular architecture of human brain metastases. *Cell* 185, 729–745.e20. <https://doi.org/10.1016/j.cell.2021.12.043>.
42. Reinecke, J.B., Jimenez Garcia, L., Gross, A.C., Cam, M., Cannon, M.V., Gust, M.J., Sheridan, J.P., Gryder, B.E., Dries, R., and Roberts, R.D. (2025). Aberrant Activation of Wound-Healing Programs within the Metastatic Niche Facilitates Lung Colonization by Osteosarcoma Cells. *Clin. Cancer Res.* 31, 414–429. <https://doi.org/10.1158/1078-0432.CCR-24-0049>.
43. Haffner, M.C., Zwart, W., Roudier, M.P., True, L.D., Nelson, W.G., Epstein, J.I., De Marzo, A.M., Nelson, P.S., and Yegnasubramanian, S. (2021). Genomic and phenotypic heterogeneity in prostate cancer. *Nat. Rev. Urol.* 18, 79–92. <https://doi.org/10.1038/s41585-020-00400-w>.
44. Hapach, L.A., Carey, S.P., Schwager, S.C., Taufalele, P.V., Wang, W., Mosier, J.A., Ortiz-Otero, N., McArdle, T.J., Goldblatt, Z.E., Lampi, M.C., et al. (2021). Phenotypic Heterogeneity and Metastasis of Breast Cancer Cells. *Cancer Res.* 81, 3649–3663. <https://doi.org/10.1158/0008-5472.CAN-20-1799>.
45. Qiu, X., Liu, Y., Shen, H., Wang, Z., Gong, Y., Yang, J., Li, X., Zhang, H., Chen, Y., Zhou, C., et al. (2021). Single-cell RNA sequencing of human femoral head in vivo. *Aging (Albany NY)* 13, 15595–15619. <https://doi.org/10.18632/aging.203124>.
46. Tirosh, I., Venteicher, A.S., Hebert, C., Escalante, L.E., Patel, A.P., Yizhak, K., Fisher, J.M., Rodman, C., Mount, C., Filbin, M.G., et al. (2016). Single-cell RNA-seq supports a developmental hierarchy in human oligodendrogloma. *Nature* 539, 309–313. <https://doi.org/10.1038/nature20123>.

47. Barkley, D., Moncada, R., Pour, M., Liberman, D.A., Dryg, I., Werba, G., Wang, W., Baron, M., Rao, A., Xia, B., et al. (2022). Cancer cell states recur across tumor types and form specific interactions with the tumor microenvironment. *Nat. Genet.* 54, 1192–1201. <https://doi.org/10.1038/s41588-022-01141-9>.
48. Kurop, M.K., Huyen, C.M., Kelly, J.H., and Blagg, B.S.J. (2021). The heat shock response and small molecule regulators. *Eur. J. Med. Chem.* 226, 113846. <https://doi.org/10.1016/j.ejmech.2021.113846>.
49. Tirosh, I., Izar, B., Prakadan, S.M., Wadsworth, M.H., 2nd, Treacy, D., Trombetta, J.J., Rotem, A., Rodman, C., Lian, C., Murphy, G., et al. (2016). Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. *Science* 352, 189–196. <https://doi.org/10.1126/science.aad0501>.
50. Ashton, T.M., McKenna, W.G., Kunz-Schughart, L.A., and Higgins, G.S. (2018). Oxidative Phosphorylation as an Emerging Target in Cancer Therapy. *Clin. Cancer Res.* 24, 2482–2490. <https://doi.org/10.1158/1078-0432.CCR-17-3070>.
51. Zhao, Z., Mei, Y., Wang, Z., and He, W. (2022). The Effect of Oxidative Phosphorylation on Cancer Drug Resistance. *Cancers (Basel)* 15, 62. <https://doi.org/10.3390/cancers15010062>.
52. Puram, S.V., Tirosh, I., Park, A.S., Patel, A.P., Yizhak, K., Gillespie, S., Rodman, C., Luo, C.L., Mroz, E.A., Emerick, K.S., et al. (2017). Single-Cell Transcriptomic Analysis of Primary and Metastatic Tumor Ecosystems in Head and Neck Cancer. *Cell* 171, 1611–1624.e24. <https://doi.org/10.1016/j.cell.2017.10.044>.
53. Li, S., Garrett-Bakelman, F.E., Chung, S.S., Sanders, M.A., Hricik, T., Rapaport, F., Patel, J., Dillon, R., Vijay, P., Brown, A.L., et al. (2016). Distinct evolution and dynamics of epigenetic and genetic heterogeneity in acute myeloid leukemia. *Nat. Med.* 22, 792–799. <https://doi.org/10.1038/nm.4125>.
54. Li, H., Courtois, E.T., Sengupta, D., Tan, Y., Chen, K.H., Goh, J.J.L., Kong, S.L., Chua, C., Hon, L.K., Tan, W.S., et al. (2017). Reference component analysis of single-cell transcriptomes elucidates cellular heterogeneity in human colorectal tumors. *Nat. Genet.* 49, 708–718. <https://doi.org/10.1038/ng.3818>.
55. Filbin, M.G., Tirosh, I., Hovestadt, V., Shaw, M.L., Escalante, L.E., Mathewson, N.D., Neftel, C., Frank, N., Pelton, K., Hebert, C.M., et al. (2018). Developmental and oncogenic programs in H3K27M gliomas dissected by single-cell RNA-seq. *Science* 360, 331–335. <https://doi.org/10.1126/science.aao4750>.
56. Robinson, D.R., Wu, Y.M., Lonigro, R.J., Vats, P., Cobain, E., Everett, J., Cao, X., Rabban, E., Kumar-Sinha, C., Raymond, V., et al. (2017). Integrative clinical genomics of metastatic cancer. *Nature* 548, 297–303. <https://doi.org/10.1038/nature23306>.
57. Dang, C.V. (2012). MYC on the path to cancer. *Cell* 149, 22–35. <https://doi.org/10.1016/j.cell.2012.03.003>.
58. Postel, E.H., Berberich, S.J., Flint, S.J., and Ferrone, C.A. (1993). Human c-myc transcription factor PuF identified as nm23-H2 nucleoside diphosphate kinase, a candidate suppressor of tumor metastasis. *Science* 261, 478–480. <https://doi.org/10.1126/science.8392752>.
59. Berberich, S.J., and Postel, E.H. (1995). PuF/NM23-H2/NDPK-B transactivates a human c-myc promoter-CAT gene via a functional nuclease hypersensitive element. *Oncogene* 10, 2343–2347.
60. Dexheimer, T.S., Carey, S.S., Zuohe, S., Gokhale, V.M., Hu, X., Murata, L.B., Maes, E.M., Weichsel, A., Sun, D., Meuliet, E.J., et al. (2009). NM23-H2 may play an indirect role in transcriptional activation of c-myc gene expression but does not cleave the nuclease hypersensitive element III(1). *Mol. Cancer Ther.* 8, 1363–1377. <https://doi.org/10.1158/1535-7163.MCT-08-1093>.
61. Bhowmick, S., and Bhowmick, N.A. (2022). RARgamma: The Bone of Contention for Endothelial Cells in Prostate Cancer Metastasis. *Cancer Res.* 82, 2975–2976. <https://doi.org/10.1158/0008-5472.CAN-22-2251>.
62. Wang, K., Jiang, L., Hu, A., Sun, C., Zhou, L., Huang, Y., Chen, Q., Dong, J., Zhou, X., and Zhang, F. (2021). Vertebral-specific activation of the CX3CL1/ICAM-1 signaling network mediates non-small-cell lung cancer spinal metastasis by engaging tumor cell-vertebral bone marrow endothelial cell interactions. *Theranostics* 11, 4770–4789. <https://doi.org/10.7150/thno.54235>.
63. Garcia-Silva, S., Benito-Martin, A., Nogue, L., Hernandez-Barranco, A., Mazariegos, M.S., Santos, V., Hergueta-Redondo, M., Ximenez-Embun, P., Kataru, R.P., Lopez, A.A., et al. (2021). Melanoma-derived small extracellular vesicles induce lymphangiogenesis and metastasis through an NGFR-dependent mechanism. *Nat. Cancer* 2, 1387–1405. <https://doi.org/10.1038/s43018-021-00272-y>.
64. Goveia, J., Rohlenova, K., Taverna, F., Treps, L., Conradi, L.C., Pircher, A., Geldhof, V., de Rooij, L.P.M.H., Kalucka, J., Sokol, L., et al. (2020). An Integrated Gene Expression Landscape Profiling Approach to Identify Lung Tumor Endothelial Cell Heterogeneity and Angiogenic Candidates. *Cancer Cell* 37, 21. <https://doi.org/10.1016/j.ccell.2019.12.001>.
65. Liu, Q., Hu, T., He, L., Huang, X., Tian, X., Zhang, H., He, L., Pu, W., Zhang, L., Sun, H., et al. (2015). Genetic targeting of sprouting angiogenesis using Aplin-CreER. *Nat. Commun.* 6, 6020. <https://doi.org/10.1038/ncomms7020>.
66. Helker, C.S., Eberlein, J., Wilhelm, K., Sugino, T., Malchow, J., Schuermann, A., Baumeister, S., Kwon, H.B., Maischein, H.M., Potente, M., et al. (2020). Apelin signaling drives vascular endothelial cells toward a pro-angiogenic state. *eLife* 9, e55589. <https://doi.org/10.7554/eLife.55589>.
67. Xie, Y., He, L., Lugano, R., Zhang, Y., Cao, H., He, Q., Chao, M., Liu, B., Cao, Q., Wang, J., et al. (2021). Key molecular alterations in endothelial cells in human glioblastoma uncovered through single-cell RNA sequencing. *JCI Insight* 6, e150861. <https://doi.org/10.1172/jci.insight.150861>.
68. Schupp, J.C., Adams, T.S., Cosme, C., Jr., Raredon, M.S.B., Yuan, Y., Omote, N., Poli, S., Chioccioli, M., Rose, K.A., Manning, E.P., et al. (2021). Integrated Single-Cell Atlas of Endothelial Cells of the Human Lung. *Circulation* 144, 286–302. <https://doi.org/10.1161/CIRCULATIONAHA.120.052318>.
69. Kusumbe, A.P., Ramasamy, S.K., and Adams, R.H. (2014). Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. *Nature* 507, 323–328. <https://doi.org/10.1038/nature13145>.
70. Viallard, C., and Larrivée, B. (2017). Tumor angiogenesis and vascular normalization: alternative therapeutic targets. *Angiogenesis* 20, 409–426. <https://doi.org/10.1007/s10456-017-9562-9>.
71. Li, X., Sun, X., and Carmeliet, P. (2019). Hallmarks of Endothelial Cell Metabolism in Health and Disease. *Cell Metab.* 30, 414–433. <https://doi.org/10.1016/j.cmet.2019.08.011>.
72. Lange, T., Valentiner, U., Wicklein, D., Maar, H., Labitzky, V., Ahlers, A.K., Starzonek, S., Genduso, S., Staffeldt, L., Pahlow, C., et al. (2022). Tumor cell E-selectin ligands determine partialefficacy of bortezomib on spontaneous lung metastasis formation of solid human tumors in vivo. *Mol. Ther.* 30, 1536–1552. <https://doi.org/10.1016/j.ymthe.2022.01.017>.
73. Kang, S.A., Hasan, N., Mann, A.P., Zheng, W., Zhao, L., Morris, L., Zhu, W., Zhao, Y.D., Suh, K.S., Dooley, W.C., et al. (2015). Blocking the adhesion cascade at the premetastatic niche for prevention of breast cancer metastasis. *Mol. Ther.* 23, 1044–1054. <https://doi.org/10.1038/mt.2015.45>.
74. Julien, S., Ivetic, A., Grigoriadis, A., QiZe, D., Burford, B., Sproviero, D., Picco, G., Gillett, C., Papp, S.L., Schaffer, L., et al. (2011). Selectin ligand sialyl-Lewis x antigen drives metastasis of hormone-dependent breast cancers. *Cancer Res.* 71, 7683–7693. <https://doi.org/10.1158/0008-5472.CAN-11-1139>.
75. Festuccia, C., Mancini, A., Gravina, G.L., Colapietro, A., Vetusch, A., Pompili, S., Ventura, L., Delle Monache, S., Iorio, R., Del Fattore, A., et al. (2019). Dual CXCR4 and E-Selectin Inhibitor, GMI-1359, Shows Anti-Bone Metastatic Effects and Synergizes with Docetaxel in Prostate

- Cancer Cell Intraosseous Growth. *Cells* 9, 32. <https://doi.org/10.3390/cells9010032>.
76. Esposito, M., Mondal, N., Greco, T.M., Wei, Y., Spadazzi, C., Lin, S.C., Zheng, H., Cheung, C., Magnani, J.L., Lin, S.H., et al. (2019). Bone vascular niche E-selectin induces mesenchymal-epithelial transition and Wnt activation in cancer cells to promote bone metastasis. *Nat. Cell Biol.* 21, 627–639. <https://doi.org/10.1038/s41556-019-0309-2>.
77. Vento-Tormo, R., Efremova, M., Botting, R.A., Turco, M.Y., Vento-Tormo, M., Meyer, K.B., Park, J.E., Stephenson, E., Polarski, K., Goncalves, A., et al. (2018). Single-cell reconstruction of the early maternal-fetal interface in humans. *Nature* 563, 347–353. <https://doi.org/10.1038/s41586-018-0698-6>.
78. Nakamura, K., Tsukasaki, M., Tsunematsu, T., Yan, M., Ando, Y., Huynh, N.C.N., Hashimoto, K., Gou, Q., Muro, R., Itabashi, A., et al. (2024). The periosteum provides a stromal defence against cancer invasion into the bone. *Nature* 634, 474–481. <https://doi.org/10.1038/s41586-024-07822-1>.
79. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* 284, 143–147. <https://doi.org/10.1126/science.284.5411.143>.
80. Pittenger, M.F., Discher, D.E., Péault, B.M., Phinney, D.G., Hare, J.M., and Caplan, A.I. (2019). Mesenchymal stem cell perspective: cell biology to clinical progress. *NPJ Regen. Med.* 4, 22. <https://doi.org/10.1038/s41536-019-0083-6>.
81. Chen, Y., Kim, J., Yang, S., Wang, H., Wu, C.J., Sugimoto, H., LeBleu, V.S., and Kalluri, R. (2021). Type I collagen deletion in alphaSMA(+) myofibroblasts augments immune suppression and accelerates progression of pancreatic cancer. *Cancer Cell* 39, 548. <https://doi.org/10.1016/j.ccell.2021.02.007>.
82. Chen, Y., Yang, S., Tavormina, J., Tampe, D., Zeisberg, M., Wang, H., Mahadevan, K.K., Wu, C.J., Sugimoto, H., Chang, C.C., et al. (2022). Oncogenic collagen I homotrimers from cancer cells bind to alpha3beta1 integrin and impact tumor microbiome and immunity to promote pancreatic cancer. *Cancer Cell* 40, 818. <https://doi.org/10.1016/j.ccell.2022.06.011>.
83. Midavaine, É., Côté, J., and Sarret, P. (2021). The multifaceted roles of the chemokines CCL2 and CXCL12 in osteophilic metastatic cancers. *Cancer Metastasis Rev.* 40, 427–445. <https://doi.org/10.1007/s10555-021-09974-2>.
84. Hao, X., Shen, Y., Chen, N., Zhang, W., Valverde, E., Wu, L., Chan, H.L., Xu, Z., Yu, L., Gao, Y., et al. (2023). Osteoprogenitor-GMP crosstalk underpins solid tumor-induced systemic immunosuppression and persists after tumor removal. *Cell Stem Cell* 30, 648–664.e8. <https://doi.org/10.1016/j.stem.2023.04.005>.
85. Perjes, A., Kilpio, T., Ulvila, J., Magga, J., Alakoski, T., Szabo, Z., Vainio, L., Halmetoja, E., Vuolteenaho, O., Petaja-Repo, U., et al. (2016). Characterization of apela, a novel endogenous ligand of apelin receptor, in the adult heart. *Basic Res. Cardiol.* 111, 2. <https://doi.org/10.1007/s00395-015-0521-6>.
86. Bai, W., Cheng, M., Jin, J., Zhang, D., Li, L., Bai, Y., and Xu, J. (2023). KAP1 modulates osteogenic differentiation via the ERK/Runx2 cascade in vascular smooth muscle cells. *Mol. Biol. Rep.* 50, 3217–3228. <https://doi.org/10.1007/s11033-022-08225-z>.
87. Wang, Y., Liu, Y., Zhang, M., Lv, L., Zhang, X., Zhang, P., and Zhou, Y. (2018). LRRRC15 promotes osteogenic differentiation of mesenchymal stem cells by modulating p65 cytoplasmic/nuclear translocation. *Stem Cell Res. Ther.* 9, 65. <https://doi.org/10.1186/s13287-018-0809-1>.
88. Wang, C.A., Hou, Y.C., Hong, Y.K., Tai, Y.J., Shen, C., Hou, P.C., Fu, J.L., Wu, C.L., Cheng, S.M., Hwang, D.Y., et al. (2025). Intercellular TIMP-1-CD63 signaling directs the evolution of immune escape and metastasis in KRAS-mutated pancreatic cancer cells. *Mol. Cancer* 24, 25. <https://doi.org/10.1186/s12943-024-02207-4>.
89. DeNardo, D.G., and Ruffell, B. (2019). Macrophages as regulators of tumour immunity and immunotherapy. *Nat. Rev. Immunol.* 19, 369–382. <https://doi.org/10.1038/s41577-019-0127-6>.
90. Goswami, S., Anandhan, S., Raychaudhuri, D., and Sharma, P. (2023). Myeloid cell-targeted therapies for solid tumours. *Nat. Rev. Immunol.* 23, 106–120. <https://doi.org/10.1038/s41577-022-00737-w>.
91. Liu, Y., Ye, G., Dong, B., Huang, L., Zhang, C., Sheng, Y., Wu, B., Han, L., Wu, C., and Qi, Y. (2022). A pan-cancer analysis of the oncogenic role of secreted phosphoprotein 1 (SPP1) in human cancers. *Ann. Transl. Med.* 10, 279. <https://doi.org/10.21037/atm-22-829>.
92. Liu, L., Zhang, R., Deng, J., Dai, X., Zhu, X., Fu, Q., Zhang, H., Tong, Z., Zhao, P., Fang, W., et al. (2022). Construction of TME and Identification of crosstalk between malignant cells and macrophages by SPP1 in hepatocellular carcinoma. *Cancer Immunol. Immunother.* 71, 121–136. <https://doi.org/10.1007/s00262-021-02967-8>.
93. Fan, H., Xu, Z., Yao, K., Zheng, B., Zhang, Y., Wang, X., Zhang, T., Li, X., Hu, H., Yue, B., et al. (2024). Osteoclast Cancer Cell Metabolic Cross-talk Confers PARP Inhibitor Resistance in Bone Metastatic Breast Cancer. *Cancer Res.* 84, 449–467. <https://doi.org/10.1158/0008-5472.CAN-23-1443>.
94. Gu, C., Chen, P., Tian, H., Yang, Y., Huang, Z., Yan, H., Tang, C., Xiang, J., Shanguan, L., Pan, K., et al. (2024). Targeting initial tumour-osteoclast spatiotemporal interaction to prevent bone metastasis. *Nat. Nanotechnol.* 19, 1044–1054. <https://doi.org/10.1038/s41565-024-01613-5>.
95. Gulati, G.S., Sikandar, S.S., Wesche, D.J., Manjunath, A., Bharadwaj, A., Berger, M.J., Ilagan, F., Kuo, A.H., Hsieh, R.W., Cai, S., et al. (2020). Single-cell transcriptional diversity is a hallmark of developmental potential. *Science* 367, 405–411. <https://doi.org/10.1126/science.aax0249>.
96. Jiao, S., Subudhi, S.K., Aparicio, A., Ge, Z., Guan, B., Miura, Y., and Sharma, P. (2019). Differences in Tumor Microenvironment Dictate T Helper Lineage Polarization and Response to Immune Checkpoint Therapy. *Cell* 179, 1177–1190.e13. <https://doi.org/10.1016/j.cell.2019.10.029>.
97. Alspach, E., Lussier, D.M., Miceli, A.P., Kizhvatov, I., DuPage, M., Luoma, A.M., Meng, W., Licht, C.F., Esaulova, E., Vomund, A.N., et al. (2019). MHC-II neoantigens shape tumour immunity and response to immunotherapy. *Nature* 574, 696–701. <https://doi.org/10.1038/s41586-019-1671-8>.
98. Oh, D.Y., and Fong, L. (2021). Cytotoxic CD4(+) T cells in cancer: Expanding the immune effector toolbox. *Immunity* 54, 2701–2711. <https://doi.org/10.1016/j.immuni.2021.11.015>.
99. Sievers, C., Craveiro, M., Friedman, J., Robbins, Y., Yang, X., Bai, K., Nguyen, A., Redman, J.M., Chari, R., Soon-Shiong, P., et al. (2023). Phenotypic plasticity and reduced tissue retention of exhausted tumor-infiltrating T cells following neoadjuvant immunotherapy in head and neck cancer. *Cancer Cell* 41, 887–902.e5. <https://doi.org/10.1016/j.ccell.2023.03.014>.
100. Crawford, A., Angelosanto, J.M., Kao, C., Doering, T.A., Odorizzi, P.M., Barnett, B.E., and Wherry, E.J. (2014). Molecular and transcriptional basis of CD4(+) T cell dysfunction during chronic infection. *Immunity* 40, 289–302. <https://doi.org/10.1016/j.immuni.2014.01.005>.
101. Zheng, C., Zheng, L., Yoo, J.K., Guo, H., Zhang, Y., Guo, X., Kang, B., Hu, R., Huang, J.Y., Zhang, Q., et al. (2017). Landscape of Infiltrating T Cells in Liver Cancer Revealed by Single-Cell Sequencing. *Cell* 169, 1342–1356.e16. <https://doi.org/10.1016/j.cell.2017.05.035>.
102. Wang, F., Long, J., Li, L., Wu, Z.X., Da, T.T., Wang, X.Q., Huang, C., Jiang, Y.H., Yao, X.Q., Ma, H.Q., et al. (2023). Single-cell and spatial transcriptome analysis reveals the cellular heterogeneity of liver metastatic colorectal cancer. *Sci. Adv.* 9, ead5464. <https://doi.org/10.1126/sciadv.adf5464>.
103. Chu, Y., Dai, E., Li, Y., Han, G., Pei, G., Ingram, D.R., Thakkar, K., Qin, J.J., Dang, M., Le, X., et al. (2023). Pan-cancer T cell atlas links a cellular stress response state to immunotherapy resistance. *Nat. Med.* 29, 1550–1562. <https://doi.org/10.1038/s41591-023-02371-y>.

104. van der Leun, A.M., Thommen, D.S., and Schumacher, T.N. (2020). CD8(+) T cell states in human cancer: insights from single-cell analysis. *Nat. Rev. Cancer* 20, 218–232. <https://doi.org/10.1038/s41568-019-0235-4>.
105. Friedrich, M.J., Neri, P., Kehl, N., Michel, J., Steiger, S., Kilian, M., Leblay, N., Maity, R., Sankowski, R., Lee, H., et al. (2023). The pre-existing T cell landscape determines the response to bispecific T cell engagers in multiple myeloma patients. *Cancer Cell* 41, 711–725.e6. <https://doi.org/10.1016/j.ccell.2023.02.008>.
106. Sun, Y., Wu, L., Zhong, Y., Zhou, K., Hou, Y., Wang, Z., Zhang, Z., Xie, J., Wang, C., Chen, D., et al. (2021). Single-cell landscape of the ecosystem in early-relapse hepatocellular carcinoma. *Cell* 184, 404–421.e16. <https://doi.org/10.1016/j.cell.2020.11.041>.
107. Miller, B.C., Sen, D.R., Al Abosy, R., Bi, K., Virkud, Y.V., LaFleur, M.W., Yates, K.B., Lako, A., Felt, K., Naik, G.S., et al. (2019). Subsets of exhausted CD8(+) T cells differentially mediate tumor control and respond to checkpoint blockade. *Nat. Immunol.* 20, 326–336. <https://doi.org/10.1038/s41590-019-0312-6>.
108. Siddiqui, I., Schaeuble, K., Chennupati, V., Fuertes Marraco, S.A., Calderon-Copete, S., Pais Ferreira, D., Carmona, S.J., Scarpellino, L., Gfeller, D., Pradervand, S., et al. (2019). Intratumoral Tcf1(+)PD-1(+)CD8(+) T Cells with Stem-like Properties Promote Tumor Control in Response to Vaccination and Checkpoint Blockade Immunotherapy. *Immunity* 50, 195–211.e10. <https://doi.org/10.1016/j.immuni.2018.12.021>.
109. Liu, Z., Zhang, Y., Ma, N., Yang, Y., Ma, Y., Wang, F., Wang, Y., Wei, J., Chen, H., Tartarone, A., et al. (2023). Progenitor-like exhausted SPY1(+)CD8(+) T cells potentiate responsiveness to neoadjuvant PD-1 blockade in esophageal squamous cell carcinoma. *Cancer Cell* 41, 1852–1870.e9. <https://doi.org/10.1016/j.ccell.2023.09.011>.
110. Bergen, V., Lange, M., Peidli, S., Wolf, F.A., and Theis, F.J. (2020). Generalizing RNA velocity to transient cell states through dynamical modeling. *Nat. Biotechnol.* 38, 1408–1414. <https://doi.org/10.1038/s41587-020-0591-3>.
111. Zhou, P., Shi, H., Huang, H., Sun, X., Yuan, S., Chapman, N.M., Connelly, J.P., Lim, S.A., Saravia, J., Kc, A., et al. (2023). Single-cell CRISPR screens in vivo map T cell fate regulomes in cancer. *Nature* 624, 154–163. <https://doi.org/10.1038/s41586-023-06733-x>.
112. Kallies, A., Zehn, D., and Utzschneider, D.T. (2020). Precursor exhausted T cells: key to successful immunotherapy? *Nat. Rev. Immunol.* 20, 128–136. <https://doi.org/10.1038/s41577-019-0223-7>.
113. Rahim, M.K., Okholm, T.L.H., Jones, K.B., McCarthy, E.E., Liu, C.C., Yee, J.L., Tamaki, S.J., Marquez, D.M., Tennooren, I., Wai, K., et al. (2023). Dynamic CD8(+) T cell responses to cancer immunotherapy in human regional lymph nodes are disrupted in metastatic lymph nodes. *Cell* 186, 1127–1143.e18. <https://doi.org/10.1016/j.cell.2023.02.021>.
114. Buttner, M., Ostner, J., Muller, C.L., Theis, F.J., and Schubert, B. (2021). scCODA is a Bayesian model for compositional single-cell data analysis. *Nat. Commun.* 12, 6876. <https://doi.org/10.1038/s41467-021-27150-6>.
115. Priestley, P., Baber, J., Lolkema, M.P., Steeghs, N., de Bruijn, E., Shale, C., Duyvesteyn, K., Haidari, S., van Hoeck, A., Onstenk, W., et al. (2019). Pan-cancer whole-genome analyses of metastatic solid tumours. *Nature* 575, 210–216. <https://doi.org/10.1038/s41586-019-1689-y>.
116. Reiter, J.G., Hung, W.T., Lee, I.H., Nagpal, S., Giunta, P., Degner, S., Liu, G., Wassenaar, E.C.E., Jeck, W.R., Taylor, M.S., et al. (2020). Lymph node metastases develop through a wider evolutionary bottleneck than distant metastases. *Nat. Genet.* 52, 692–700. <https://doi.org/10.1038/s41588-020-0633-2>.
117. Arriaga, J.M., Panja, S., Alshalalfa, M., Zhao, J., Zou, M., Giacobbe, A., Madubata, C.J., Kim, J.Y., Rodriguez, A., Coleman, I., et al. (2020). A MYC and RAS co-activation signature in localized prostate cancer drives bone metastasis and castration resistance. *Nat. Cancer* 1, 1082–1096. <https://doi.org/10.1038/s43018-020-00125-0>.
118. Qiu, X., Boufaied, N., Hallal, T., Feit, A., de Polo, A., Luoma, A.M., Alahmadi, W., Larocque, J., Zadra, G., Xie, Y., et al. (2022). MYC drives aggressive prostate cancer by disrupting transcriptional pause release at androgen receptor targets. *Nat. Commun.* 13, 2559. <https://doi.org/10.1038/s41467-022-30257-z>.
119. Puts, G.S., Leonard, M.K., Pamidimukkala, N.V., Snyder, D.E., and Kaetzel, D.M. (2018). Nuclear functions of NME proteins. *Lab. Invest.* 98, 211–218. <https://doi.org/10.1038/labinvest.2017.109>.
120. Sengupta, A., Roy, S.S., and Chowdhury, S. (2021). Non-duplex G-Quadruplex DNA Structure: A Developing Story from Predicted Sequences to DNA Structure-Dependent Epigenetics and Beyond. *Acc. Chem. Res.* 54, 46–56. <https://doi.org/10.1021/acs.accounts.0c00431>.
121. Qi, Y., Wei, J., and Zhang, X. (2021). Requirement of transcription factor NME2 for the maintenance of the stemness of gastric cancer stem-like cells. *Cell Death Dis.* 12, 924. <https://doi.org/10.1038/s41419-021-04234-1>.
122. Wen, S., Wang, X., Wang, Y., Shen, J., Pu, J., Liang, H., Chen, C., Liu, L., and Dai, P. (2018). Nucleoside diphosphate kinase 2 confers acquired 5-fluorouracil resistance in colorectal cancer cells. *Artif. Cells, Nanomed. Biotechnol.* 46, 896–905. <https://doi.org/10.1080/21691401.2018.1439835>.
123. Gong, Y., Yang, G., Wang, Q., Wang, Y., and Zhang, X. (2020). NME2 Is a Master Suppressor of Apoptosis in Gastric Cancer Cells via Transcriptional Regulation of miR-100 and Other Survival Factors. *Mol. Cancer Res.* 18, 287–299. <https://doi.org/10.1158/1541-7786.MCR-19-0612>.
124. McEver, R.P. (2015). Selectins: initiators of leucocyte adhesion and signalling at the vascular wall. *Cardiovasc. Res.* 107, 331–339. <https://doi.org/10.1093/cvr/cvv154>.
125. Melzer, C., von der Ohe, J., Lehnert, H., Ungefroren, H., and Hass, R. (2017). Cancer stem cell niche models and contribution by mesenchymal stroma/stem cells. *Mol. Cancer* 16, 28. <https://doi.org/10.1186/s12943-017-0595-x>.
126. Widder, M., Lutzendorf, J., Caysa, H., Unverzagt, S., Wickenhauser, C., Benndorf, R.A., Schmoll, H.J., Muller-Tidow, C., Muller, T., and Muller, L.P. (2016). Multipotent mesenchymal stromal cells promote tumor growth in distinct colorectal cancer cells by a beta1-integrin-dependent mechanism. *Int. J. Cancer* 138, 964–975. <https://doi.org/10.1002/ijc.29844>.
127. Li, M., Wang, J., Wang, C., Xia, L., Xu, J., Xie, X., and Lu, W. (2020). Micro-environment remodeled by tumor and stromal cells elevates fibroblast-derived COL1A1 and facilitates ovarian cancer metastasis. *Exp. Cell Res.* 394, 112153. <https://doi.org/10.1016/j.yexcr.2020.112153>.
128. Lowery, J.W., and Rosen, V. (2018). The BMP Pathway and Its Inhibitors in the Skeleton. *Physiol. Rev.* 98, 2431–2452. <https://doi.org/10.1152/physrev.00028.2017>.
129. Kamizaki, K., Endo, M., Minami, Y., and Kobayashi, Y. (2021). Role of noncanonical Wnt ligands and Ror-family receptor tyrosine kinases in the development, regeneration, and diseases of the musculoskeletal system. *Dev. Dyn.* 250, 27–38. <https://doi.org/10.1002/dvdy.151>.
130. Vivic, I., and Belev, B. (2021). The pathogenesis of bone metastasis in solid tumors: a review. *Croat. Med. J.* 62, 270–282. <https://doi.org/10.3325/cmj.2021.62.270>.
131. Koppula, P., Zhuang, L., and Gan, B. (2021). Cystine transporter SLC7A11/xCT in cancer: ferroptosis, nutrient dependency, and cancer therapy. *Protein Cell* 12, 599–620. <https://doi.org/10.1007/s13238-020-00789-5>.
132. Avnet, S., Di Pompo, G., Lemma, S., and Baldini, N. (2019). Cause and effect of microenvironmental acidosis on bone metastases. *Cancer Metastasis Rev.* 38, 133–147. <https://doi.org/10.1007/s10555-019-09790-9>.
133. Piao, X., Wu, X., Yan, Y., Li, Y., Li, N., Xue, L., and He, F. (2024). Targeting EZH2 attenuates the ferroptosis-mediated osteoblast-osteoclast imbalance in rheumatoid arthritis. *Int. Immunopharmacol.* 143, 113201. <https://doi.org/10.1016/j.intimp.2024.113201>.
134. Coon, B.G., Burgner, J., Camonis, J.H., and Aguilar, R.C. (2010). The epsin family of endocytic adaptors promotes fibrosarcoma migration and

- p>invasion.
- J. Biol. Chem.*
- 285, 33073–33081.
- <https://doi.org/10.1074/jbc.M110.124123>
- .
135. Kwak, T., Wang, F., Deng, H., Condamine, T., Kumar, V., Perego, M., Kossenkova, A., Montaner, L.J., Xu, X., Xu, W., et al. (2020). Distinct Populations of Immune-Suppressive Macrophages Differentiate from Monocytic Myeloid-Derived Suppressor Cells in Cancer. *Cell Rep.* 33, 108571. <https://doi.org/10.1016/j.celrep.2020.108571>.
 136. Zhang, L., Li, Z., Skrzypczynska, K.M., Fang, Q., Zhang, W., O'Brien, S.A., He, Y., Wang, L., Zhang, Q., Kim, A., et al. (2020). Single-Cell Analyses Inform Mechanisms of Myeloid-Targeted Therapies in Colon Cancer. *Cell* 181, 442–459.e29. <https://doi.org/10.1016/j.cell.2020.03.048>.
 137. Qi, J., Sun, H., Zhang, Y., Wang, Z., Xun, Z., Li, Z., Ding, X., Bao, R., Hong, L., Jia, W., et al. (2022). Single-cell and spatial analysis reveal interaction of FAP(+) fibroblasts and SPP1(+) macrophages in colorectal cancer. *Nat. Commun.* 13, 1742. <https://doi.org/10.1038/s41467-022-29366-6>.
 138. Zhu, Y.J., Chang, X.S., Zhou, R., Chen, Y.D., Ma, H.C., Xiao, Z.Z., Qu, X., Liu, Y.H., Liu, L.R., Li, Y., et al. (2022). Bone metastasis attenuates efficacy of immune checkpoint inhibitors and displays “cold” immune characteristics in Non-small cell lung cancer. *Lung Cancer* 166, 189–196. <https://doi.org/10.1016/j.lungcan.2022.03.006>.
 139. Qin, A., Zhao, S., Miah, A., Wei, L., Patel, S., Johns, A., Grogan, M., Bertino, E.M., He, K., Shields, P.G., et al. (2021). Bone Metastases, Skeletal-Related Events, and Survival in Patients With Metastatic Non-Small Cell Lung Cancer Treated With Immune Checkpoint Inhibitors. *J. Natl. Compr. Canc. Netw.* 19, 915–921. <https://doi.org/10.6004/jnccn.2020.7668>.
 140. Ales, E., and Sackstein, R. (2023). The biology of E-selectin ligands in leukemogenesis. *Adv. Cancer Res.* 157, 229–250. <https://doi.org/10.1016/bs.acr.2022.07.001>.
 141. Toriumi, K., Onodera, Y., Takehara, T., Mori, T., Hasei, J., Shigi, K., Iwakaki, N., Ozaki, T., Akagi, M., Nakanishi, M., and Teramura, T. (2023). LRRC15 expression indicates high level of stemness regulated by TWIST1 in mesenchymal stem cells. *iScience* 26, 106946. <https://doi.org/10.1016/j.isci.2023.106946>.
 142. Zhu, X., You, S., Du, X., Song, K., Lv, T., Zhao, H., and Yao, Q. (2023). LRRC superfamily expression in stromal cells predicts the clinical prognosis and platinum resistance of ovarian cancer. *BMC Med. Genomics* 16, 10. <https://doi.org/10.1186/s12920-023-01435-9>.
 143. Krishnamurthy, A.T., Shyer, J.A., Thai, M., Gandham, V., Buechler, M.B., Yang, Y.A., Pradhan, R.N., Wang, A.W., Sanchez, P.L., Qu, Y., et al. (2022). LRRC15(+) myofibroblasts dictate the stromal setpoint to suppress tumour immunity. *Nature* 611, 148–154. <https://doi.org/10.1038/s41586-022-05272-1>.
 144. Zheng, G.X.Y., Terry, J.M., Belgrader, P., Ryvkin, P., Bent, Z.W., Wilson, R., Ziraldo, S.B., Wheeler, T.D., McDermott, G.P., Zhu, J., et al. (2017). Massively parallel digital transcriptional profiling of single cells. *Nat. Commun.* 8, 14049. <https://doi.org/10.1038/ncomms14049>.
 145. Young, M.D., and Behjati, S. (2020). SoupX removes ambient RNA contamination from droplet-based single-cell RNA sequencing data. *GigaScience* 9, g1aa151. <https://doi.org/10.1093/gigascience/giaa151>.
 146. McGinnis, C.S., Murrow, L.M., and Gartner, Z.J. (2019). DoubletFinder: Doublet Detection in Single-Cell RNA Sequencing Data Using Artificial Nearest Neighbors. *Cell Syst.* 8, 329–337.e4. <https://doi.org/10.1016/j.cels.2019.03.003>.
 147. Wolf, F.A., Angerer, P., and Theis, F.J. (2018). SCANPY: large-scale single-cell gene expression data analysis. *Genome Biol.* 19, 15. <https://doi.org/10.1186/s13059-017-1382-0>.
 148. Patel, A.P., Tirosh, I., Trombetta, J.J., Shalek, A.K., Gillespie, S.M., Wakimoto, H., Cahill, D.P., Nahed, B.V., Curry, W.T., Martuza, R.L., et al. (2014). Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science* 344, 1396–1401. <https://doi.org/10.1126/science.1254257>.
 149. Kotliar, D., Veres, A., Nagy, M.A., Tabrizi, S., Hodis, E., Melton, D.A., and Sabeti, P.C. (2019). Identifying gene expression programs of cell-type identity and cellular activity with single-cell RNA-Seq. *eLife* 8, e43803. <https://doi.org/10.7554/eLife.43803>.
 150. Zhou, Y., Zhou, B., Pache, L., Chang, M., Khodabakhshi, A.H., Tanaseichuk, O., Benner, C., and Chanda, S.K. (2019). Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat. Commun.* 10, 1523. <https://doi.org/10.1038/s41467-019-09234-6>.
 151. Kersevcicute, I., and Gordevicius, J. (2023). aPEAR: an R package for autonomous visualization of pathway enrichment networks. *Bioinformatics* 39, btad672. <https://doi.org/10.1093/bioinformatics/btad672>.
 152. Angerer, P., Haghverdi, L., Büttner, M., Theis, F.J., Marr, C., and Büttner, F. (2016). destiny: diffusion maps for large-scale single-cell data in R. *Bioinformatics* 32, 1241–1243. <https://doi.org/10.1093/bioinformatics/btv715>.
 153. Aibar, S., González-Blas, C.B., Moerman, T., Huynh-Thu, V.A., Imrichova, H., Hulselmans, G., Rambow, F., Marine, J.C., Geurts, P., Aerts, J., et al. (2017). SCENIC: single-cell regulatory network inference and clustering. *Nat. Methods* 14, 1083–1086. <https://doi.org/10.1038/nmeth.4463>.
 154. Polanski, K., Young, M.D., Miao, Z., Meyer, K.B., Teichmann, S.A., and Park, J.E. (2020). BBKNN: fast batch alignment of single cell transcriptomes. *Bioinformatics* 36, 964–965. <https://doi.org/10.1093/bioinformatics/btz625>.
 155. Satija, R., Farrell, J.A., Gennert, D., Schier, A.F., and Regev, A. (2015). Spatial reconstruction of single-cell gene expression data. *Nat. Biotechnol.* 33, 495–502. <https://doi.org/10.1038/nbt.3192>.
 156. La Manno, G., Soldatov, R., Zeisel, A., Braun, E., Hochgerner, H., Petukhov, V., Lidschreiber, K., Kastrioti, M.E., Lönnerberg, P., Furlan, A., et al. (2018). RNA velocity of single cells. *Nature* 560, 494–498. <https://doi.org/10.1038/s41586-018-0414-6>.
 157. Dimitrov, D., Türei, D., Garrido-Rodriguez, M., Burmedi, P.L., Nagai, J.S., Boys, C., Ramirez Flores, R.O., Kim, H., Szalai, B., Costa, I.G., et al. (2022). Comparison of methods and resources for cell-cell communication inference from single-cell RNA-Seq data. *Nat. Commun.* 13, 3224. <https://doi.org/10.1038/s41467-022-30755-0>.
 158. Zunder, E.R., Finck, R., Behbehani, G.K., Amir, E.A.D., Krishnaswamy, S., Gonzalez, V.D., Lorange, C.G., Bjornson, Z., Spitzer, M.H., Bodenmiller, B., et al. (2015). Palladium-based mass tag cell barcoding with a doublet-filtering scheme and single-cell deconvolution algorithm. *Nat. Protoc.* 10, 316–333. <https://doi.org/10.1038/nprot.2015.020>.
 159. Finck, R., Simonds, E.F., Jager, A., Krishnaswamy, S., Sachs, K., Fantl, W., Pe'er, D., Nolan, G.P., and Bendall, S.C. (2013). Normalization of mass cytometry data with bead standards. *Cytometry. A* 83, 483–494. <https://doi.org/10.1002/cyto.a.22271>.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD45	Biolegend	Cat# 103102; RRID: AB_312967
CD3e	Biolegend	Cat# 100302; RRID: AB_312667
CD192(CCR2)	RD	Cat# MAB55381-100; RRID: AB_2749828
MHC II(I-A/I-E)	Biolegend	Cat# 107602; RRID: AB_313317
Granzyme B	Biolegend	Cat# 372202; RRID: AB_2686929
CD117(c-kit)	Biolegend	Cat# 105802; RRID: AB_313211
CD69	Biolegend	Cat# 104502; RRID: AB_313105
CD279(PD-1)	Biolegend	Cat# 135202; RRID: AB_1877121
CD161(NK-1.1)	Biolegend	Cat# 108702; RRID: AB_313389
Ly-6C	Biolegend	Cat# 128002; RRID: AB_1134214
CD64(FcγRI)	Biolegend	Cat# 139302; RRID: AB_10613107
CD44	Biolegend	Cat# 103002; RRID: AB_312953
CD25	Biolegend	Cat# 101902; RRID: AB_312845
CD11c	Biolegend	Cat# 117302; RRID: AB_313771
CD19	Biolegend	Cat# 115502; RRID: AB_313637
Ki-67	eB	Cat# 14-5698-82; RRID: AB_10854564
CD103	Biolegend	Cat# 121402; RRID: AB_535945
CD194(CCR4)	Biolegend	Cat# 131202; RRID: AB_1227524
CD83	Biolegend	Cat# 121502; RRID: AB_572023
CD86	Biolegend	Cat# 105002; RRID: AB_313145
F4/80	Biorad	Cat# MCA497G; RRID: AB_872005
CD62L	Biolegend	Cat# 104402; RRID: AB_313089
TIGIT(VSTM3)	RD	Cat# MAB72671; RRID:AB_3658752
CD206(MMR)	Biolegend	Cat# 141702; RRID:AB_10900233
Ly-6G	Biolegend	Cat# 127602; RRID:AB_1089180
CD39	Biolegend	Cat# 135702; RRID:AB_2099922
T-bet	Biolegend	Cat# 644802; RRID:AB_1595503
CD27	Biolegend	Cat# 124202; RRID:AB_1236456
CD278(ICOS)	Biolegend	Cat# 313502; RRID:AB_416326
FOXP3	eB	Cat# 14-5773-82; RRID:AB_467576
CD47	Biolegend	Cat# 127502; RRID:AB_1089035
MERTK(Mer)	Biolegend	Cat# 151502; RRID: AB_2566624
CX3CR1	Biolegend	Cat# 149002; RRID:AB_2564313
CD127(IL-7Rα)	Biolegend	Cat# 135002; RRID:AB_1937287
CD172a(SIRPα)	Biolegend	Cat# 144002; RRID:AB_11203711
CD183(CXCR3)	Biolegend	Cat# 126502; RRID:AB_1027635
TCR β chain	Biolegend	Cat# 109202; RRID:AB_313425
CD366(Tim-3)	Biolegend	Cat# 119702; RRID:AB_345376
CD4	Biolegend	Cat# 100576; RRID:AB_2832266
CD8a	Biolegend	Cat# 100746; RRID:AB_11147171
CD11b	Biolegend	Cat# 101202; RRID:AB_312785
EpCAM	Abcam	Cat# ab213500; RRID:AB_2884975
SPP1	Abcam	Cat# ab214050; RRID:AB_2894860
CD31	Abcam	Cat# ab182981; RRID:AB_2920881

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
CD8	Abcam	Cat# ab217344; RRID:AB_2890649
CD68	CST	Cat# 76437; RRID: AB_2799882
CD44	CST	Cat# 37259; RRID: AB_2750879
Perforin	CST	Cat# 31647; RRID: AB_2857978
Granzyme B	CST	Cat# 46890; RRID: AB_2799313
Cleaved Caspase-3	CST	Cat# 9661; RRID:AB_2341188
SELP	Proteintech	Cat# 60322-1-Ig; RRID:AB_2881433
APLNR	Proteintech	Cat# 20341-1-AP; RRID:AB_2878676
COL1A1	Proteintech	Cat# 67288-1-Ig; RRID:AB_2882554
ITGB1	Proteintech	Cat# 26918-1-AP; RRID:AB_2880685
THY1	Proteintech	Cat# 66766-1-Ig; RRID:AB_2882112
NME2	Proteintech	Cat# 20493-1-AP; RRID:AB_10695634
c-MYC	Proteintech	Cat# 67447-1-Ig; RRID:AB_2882681
TNF- α	Affinity Biosciences	Cat# AF7014; RRID:AB_2835319
IFN- γ	Affinity Biosciences	Cat# DF6045; RRID:AB_2838015
Cleaved Caspase-9	Affinity Biosciences	Cat# AF5240; RRID:AB_2837726
SELE	Santa Cruz Biotechnology	Cat# sc-137054; RRID:AB_2186681
Ki67	HUABIO	Cat# HA721115; RRID:AB_3072239
Chemicals, peptides, and recombinant proteins		
mouse IgG1	selleck	Cat# A2123
anti-TIGIT mAb Tiragolumab	selleck	Cat# A2144
anti-PD-1 mAb	selleck	Cat# A2122
Critical commercial assays		
IHC kit	Zhongshanjinqiao	Cat# PV-6000
TRAP kit	Saiweier Biotechnology	Cat# G1050
Opal Multiplex IHC kit	Akoya Biosciences	Cat# NEL861001KT
Deposited data		
Pan-cancer bone metastases	This paper	NODE: OEP005136, https://www.biosino.org/node/project/detail/OEP005136
Human brain metastases	Gonzalez H et al. ⁴¹	GEO: GSE186344
Breast carcinoma	Azizi E et al. ²⁸	GEO: GSE114725
Colon adenocarcinoma	Pelka K et al. ²⁹	GEO: GSE178341
Colon adenocarcinoma	Che LH et al. ³⁰	GEO: GSE178318
Colon adenocarcinoma	Liu Y et al. ³¹	GEO: GSE164522
Colon adenocarcinoma	Lenos KJ et al. ³²	GEO: GSE183916
Hepatocellular carcinoma	Lu Y et al. ³³	GEO: GSE149614
Lung carcinoma	Zhang, X et al. ²⁵	EMBL-EBI: PRJNA1129208
Lung carcinoma	Kim N et al. ³⁴	GEO: GSE131907
Nasopharyngeal carcinoma	Liu Y et al. ³⁵	GEO: GSE162025
Osteosarcoma	Reinecke JB et al. ⁴²	GEO: GSE270231
Prostate adenocarcinoma	Kfoury Y et al. ²⁶	GEO: GSE143791
Prostate adenocarcinoma	Chen S et al. ³⁶	GEO: GSE141445
Renal carcinoma	Zheng L et al. ²³	GEO: GSE156728
Renal carcinoma	Mei S et al. ²⁷	GEO: GSE202813
Renal carcinoma	Ma F et al. ³⁸	NODE: OEP004678
Stomach adenocarcinoma	Kang B et al. ³⁷	GEO: GSE206785
Thyroid carcinoma	Zheng L et al. ²³	GEO: GSE156728

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Healthy bone	Oetjen KA et al. ³⁹	GEO: GSE120221
Healthy bone	Bandyopadhyay S et al. ⁴⁰	GEO: GSE253355
Healthy bone	Liu Y et al. ⁴⁵	GEO: GSE147390
MET500 datasets	Robinson DR et al. ⁵⁶	https://met500.med.umich.edu/downloadMet500DataSets
Experimental models: Cell lines		
4T1(Mouse breast carcinoma)	ATCC	Cat# CRL-2539; RRID:CVCL_0125
Software and algorithms		
CellRanger version 6.0.2	Zheng GX et al. ¹⁴⁴	https://www.10xgenomics.com/support/software/cell-ranger
SoupX version 1.5.2	Young MD et al. ¹⁴⁵	https://github.com/constantAmateur/SoupX
DoubletFinder version 2.0.3	McGinnis CS et al. ¹⁴⁶	https://github.com/chris-mcginis-ucsf/DoubletFinder
Scanpy version 1.8.2	Wolf FA et al. ¹⁴⁷	https://github.com/scverse/scanpy
InferCNV version 1.3.3	Patel AP et al. ¹⁴⁸	https://github.com/broadinstitute/inferCNV
cNMF version 1.6	Kotliar D et al. ¹⁴⁹	https://github.com/dylkot/cNMF
Metascape version 3.5	Zhou Y et al. ¹⁵⁰	https://metascape.org/
aPEAR version 1.0	Kerseviciute I et al. ¹⁵¹	https://github.com/kerseviciute/aPEAR
destiny version 2.14.0	Angerer P et al. ¹⁵²	https://github.com/theislab/destiny
velocity version 0.17.17	Bergen V et al. ¹¹⁰	https://velocityto.org/
cytoTRACE version 3.0.0	Gulati GS et al. ⁹⁵	https://github.com/gunsagargulati/CytoTRACE
CellPhoneDB version 2.0.0	Vento-Tormo R et al. ⁷⁷	https://github.com/ventolab/CellphoneDB
pySCENIC version 0.11.2	Aibar S et al. ¹⁵³	https://github.com/aertslab/SCENIC

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human participants

53 human specimens from 52 patients (2 specimens were obtained from a same patient) were pathologically diagnosed with bone tumors, including 53 bone metastases. Their ages ranged from 34 to 77, with a median age of 60 years 18 of the cases were women and 34 men. Twenty-five patients received chemotherapy, radiotherapy, targeted therapy or immunotherapy before surgery, and the remaining 27 patients received no treatment. Until follow-up deadline, the time to death since initial bone metastases diagnosis ranged from 18 days to 993 days, with a median survival 634 days. Anatomically, 44 cases were located in different segments of the spine, and the rest were located in the pelvis, femur/humerus, ribs, scapula and so on. Fresh samples isolated from patients in operation were cut into a range of 0.2–1.0 g small pieces for further single cell RNA-seq, and the remaining tissues were formalin-fixed and paraffin-embedded were used for immunohistochemical staining. We also collected 6 paired primary and bone metastatic samples as well as 4 paired bone metastases and adjacent normal bone samples from the same patients (2 prostate cancer patients; 2 female lung cancer patients) for scRNA-seq analysis. Moreover, nine bone metastasis samples from diverse primary origins were collected for mIHC, IHC, and TRAP staining. Notably, two of these samples derived from lung adenocarcinoma were collected additionally with their paired lung primary lesions and brain metastatic lesions. The clinical characteristics of these patients are available and summarized in Table S1. All samples were enrolled in this study after approvals by the Ethics Committee of Fudan University Shanghai Cancer Center, IRB Number 050432-4-2108*. Informed consent was obtained from all patients in this study.

Experimental bone metastases

Female athymic BALB/c mice (6 weeks old) were obtained from Gempharmatech Co. Ltd. and maintained under specific pathogen-free conditions. The Institutional Animal Care and Use Committee (IACUC) of Fudan University Shanghai Cancer Center reviewed and approved all animal experiments. 4T1 cells were acquired from the American Type Culture Collection (ATCC). We expanded a single clonal 4T1 cells expressing luciferase, cells were harvested, washed and re-suspended in sterile phosphate buffered saline (PBS). To establish a bone metastasis model, $5 \times 10^4/20 \mu\text{L}$ 4T1 cells stable expressing luciferase were injected into the iliac artery of female BALB/c mice. The 4T1 bone metastasis model of BALB/c mice were randomly divided into 4 treatment groups ($n = 9$): I. control group (mouse IgG1 10mg/kg.i.p. every other day, selleck A2123); II. TIGIT group (anti-TIGIT mAb Tiragolumab 10mg/kg.i.p., every other day, selleck A2144); III. PD-1 group (anti-PD-1 mAb20mg/kg i.p., selleck A2122); IV. PD-1+TIGIT group (anti-TIGIT mAb Tiragolumab 10mg/kg and anti-PD-1 mAb20mg/kg). At 17 days after the injection, the fresh bone metastases samples ($n = 3$) were isolated from the above mice and preserved in MACS Tissue Storage Solution (Miltenyi Biotec) at 4°C for CyTOF. The remaining tissues of these four groups were imaged and analyzed using the IVIS Spectrum imaging system (Lumina III, PerkinElmer, USA) and microCT

at 20 days after the injection. After the scan, the image reconstruction was analyzed. The bones were extracted and sectioned with a diamond knife after imaging. The sections were stained for immunohistochemistry (IHC) and tartrate-resistant acid phosphatase (TRAP) staining.

METHOD DETAILS

Sample collection and single cell RNA-seq library preparations

Fresh samples were preserved in MACS Tissue Storage Solution (Miltenyi Biotec) at 4°C after surgery. Tumor tissues were cut into a range of 0.2–1.0 g small pieces and dissociated in 5 mL enzyme mix containing 4.7 mL RPMI 1640 (Gibco), 200 μ L Enzyme H, 100 μ L Enzyme R and 25 μ L Enzyme A (Miltenyi Biotec, MACS Tumor Dissociation Kit, human). The samples were subsequently incubated in a 37°C thermostatic shaker for 35 min. Then suspended samples were filtered through a 40- μ m Cell-Strainer nylon mesh (BD) with 30 mL of RPMI 1640 and centrifuged at 300 $\times$ g for 7 min. After removing the supernatant, we used Red Blood Cell Lysis Solution (Miltenyi Biotec # 130-094-183) and the Dead Cell Removal Kit (Miltenyi Biotec # 130-090-101) to remove red blood cells and obtain live cells. Cell suspension was centrifuged at 300 $\times$ g for 7 min and the pellet was re-suspended in 1 mL PBS solution. Once the desired cell suspension was obtained, the sample was immediately placed on ice for subsequent GEMs preparation and reverse transcription. The single cell libraries were prepared according to the standard protocols and sequenced on Illumina NovaSeq 6000 Systems using paired-end sequencing (150 bp in length).

Histology

Tissue Preparation and Processing: human tumor tissues were fixed in 10% formalin and embedded in paraffin. For mouse-derived tibial bone tumor tissues, samples were excised, fixed in 4% paraformaldehyde (PFA) for 3 days, decalcified in 10% EDTA for 2 weeks, and subsequently paraffin-embedded. All tissues were sectioned into 4- μ m-thick slices using a microtome.

A standard IHC kit (Zhongshanjinjiao, PV-6000) was employed for IHC following the manufacturer's instructions. Specifically, the sections were deparaffinized and dehydrated using xylene and graded alcohols, followed by rehydration with demineralized water. To retrieve the antigens, the tissue sections were placed in boiled antigen retrieval buffer for 5 min. After that, the sections were incubated with a 3% hydrogen peroxide (H₂O₂) solution for 15 min at room temperature in the dark. For immunostaining, the sections were treated with primary antibodies followed by incubation with secondary antibodies and DAB detection. The sections were finally imaged using a digitalized microscope camera. For quantitative analysis, three randomly-chosen fields of view were captured per mouse sample. Immunoreactivity scores (IRS) were calculated as the product of staining intensity and positive cell proportion. The staining intensity was scored as: 0 (no staining), 1 (light yellow), 2 (yellow brown), and 3 (brown). The proportion of positive tumor cells was scored as: 0 (no positive tumor cells), 1 (70% positive tumor cells). The IHC-score was recorded based on the mean value of the selected fields.

TRAP staining was conducted using a TRAP kit (Saiweier Biotechnology, G1050) according to manufacturer's protocol. The area of TRAP-positive cells was quantified with the help of ImageJ software.

Multiplex immunofluorescence

Multiplex staining of FFPE tissue was performed using the Opal Multiplex IHC kit (Akoya Biosciences, NEL861001KT) according to manufacturer's instruction. Briefly, different primary antibodies (see [key resources table](#)) were sequentially applied, followed by HRP-conjugated secondary antibody incubation and tyramide signal amplification. The sections were microwave heat-treated after each TSA operation. Nuclei were stained with DAPI after all the human antigens had been labeled. Finally, the slides were mounted with mounting medium, and immunofluorescence microscopy images of the cells were obtained via the Vectra Polaris Automated Quantitative Pathology Imaging System (Akoya Biosciences) and processed with advanced HALO Image Analysis.

Spatial Proximity Analysis: to explore the spatial proximity between tumor cells and other cells, whole-tissue scans were segmented to identify ITGB1⁺ tumor cells (ITGB1⁺ EpCAM⁺), COL1A1⁺ MSCs (COL1A1⁺ THY1⁺), CD44⁺ tumor cells (CD44⁺ EpCAM⁺), and SPP1⁺ macrophages (SPP1⁺ CD68⁺) using HALO Cell Classification algorithms. The Proximity Analysis module was then applied to quantify: (1) ITGB1⁺ Tumor Cell-COL1A1⁺ MSCs Interactions: the numbers of ITGB1⁺ tumor cells (ITGB1⁺ EpCAM⁺) located within a specific distance range (0–20 μ m; 50–200 μ m) from COL1A1⁺ MSCs (COL1A1⁺ THY1⁺); (2) CD44⁺ Tumor Cell-CD68⁺ Macrophage Interactions: the numbers of CD44⁺ tumor cells (CD44⁺ EpCAM⁺) located within a specific distance range (0–20 μ m; 50–200 μ m) from SPP1⁺ macrophages (SPP1⁺ CD68⁺). The antibodies used in immunohistochemistry and Multi-spectral immunohistochemistry were collected in [key resources table](#).

Mass cytometry staining

Tissues were cut into 1 mm³ pieces and digested. Filter the dissociated tissue through the 70 μ m cell strainer. Collect the cells by centrifugation at 300 $\times$ g for 5 min at 2°C–8°C. Aspirate the supernatant and resuspend the cells in CSB and count cell number. The cells are ready for further staining. Label single cell suspension using 194Pt for 5 min to distinguish live or dead. Add Fc blocking mix for 20 min to block the FcR-involved unwanted staining. Surface marker staining using surface antibodies mix for 30 min. DNA Intercalator-Ir overnight staining for discrimination of dead cells from live cells or to discriminate single nucleated cells from doublets. Intracellular marker staining using intracellular or nucleus antibodies mix for 30 min. Sample barcoding using unique barcode isotope

combination for 30 min. Resuspend the cells into DI water, ready for running on CyTOF. Run a Tuning and QC procedures to calibrate the CyTOF instrument. Mix EQ Four Element Calibration Beads into barcoded samples. Load the mixed samples to collect cell data. The antibodies used in mass cytometry were collected in [key resources table](#). The markers are organized in [Table S3](#).

QUANTIFICATION AND STATISTICAL ANALYSIS

Single-cell RNA-seq data processing

Raw sequencing reads were aligned to the GRCh38 human reference genome and quantified gene expression count using Cell Ranger (version 6.0.2),¹⁴⁴ default parameters for read trimming, filtering, and gene annotation. The output was processed using SoupX (version 1.5.2)¹⁴⁵ to remove cell-free RNA. The soupQuantile parameter was set to 0.15. DoubletFinder (version 2.0.3)¹⁴⁶ was used to predict potential doublets based on the expected doublet rate derived from the loading rate, which were excluded from further analysis. The pN parameter was set to 0.25. Then, identify the optimal pK parameter by running the summarizeSweep function followed by the find.pKfunction. Further low quality cells that characterized with less than 500, or more than 9,000 genes or more than 10% mitochondrial genes count were excluded from further analyses. Genes that were expressed in less than 3 cells also were filtered.

Batch effect correction and unsupervised clustering

The Scanpy (version 1.8.2)¹⁴⁷ pipeline was applied to dimension reduction and unsupervised clustering. After quality control, library-size correction method was used for each cell to normalize raw count by using *scanpy.pp.normalize_total* function in Scanpy. The logarithmized normalized count matrix was used for the downstream analysis. The top 2,000 highly variable genes were selected by variance stabilizing transformation method with *scanpy.pp.highly_variable_genes* function. Then the total count per cell, the percentage of mitochondrial gene count, heat shock protein associated genes count and ribosomal proteins count, and cell cycle genes were regressed out by using *scanpy.pp.regress_out* function. All variably-expressed genes were scaled and used to perform principal component analysis. 100 principal components were calculated to reveal the main axes of variation by using *scanpy.tl.pca* function. Due to the heterogeneity of tumor cells, debatching is not suitable. For all cells including cancer cells, principal components covering the highest variance in the dataset were selected based on elbow and Jackstraw plots. For stromal cells, bbknn algorithm¹⁵⁴ was applied to remove batch effect from different samples. Then, cells were clustered by unsupervised graph-based clustering algorithm using their expression profiles with *scanpy.tl.leiden* function. Cells were performed down scaling with Uniform Manifold Approximation and Projection (UMAP) implemented in *scanpy.tl.umap* function for visualization. The cluster-specific marker genes were identified with *scanpy.tl.rank_genes_groups* function. All parameters not explicitly specified in the analysis were set to their default values.

Multiple dataset integration

This study integrated single-cell transcriptomic datasets from public repositories (see [key resources table](#)), encompassing diverse primary tumors, metastatic lesions (including hepatic and brain metastases et al.), and normal bone tissue control samples. To eliminate alignment biases arising from heterogeneous experimental workflows, all integrated datasets in this study were generated exclusively using the Chromium Single-Cell 3' or 5' Library Construction Systems (10X Genomics). Each single-cell count matrix underwent rigorous and standardized quality control to detect and filter out low-quality cells/samples or potential confounding factors. Subsequently, we integrated samples across diverse tissues and research cohorts. During integration, to achieve precise cell type annotation while maintaining cross-dataset consistency, we performed initial major cell lineage labeling on individual datasets. Following this, major cell classes were extracted and subjected to fine-grained subtyping using the *sc.tl.ingest* method. Complete methodological details for the Scanpy-based ingest integration framework employed in this study are publicly accessible via step-by-step online tutorials (<https://scanpy.readthedocs.io/en/stable/generated/scanpy.tl.ingest.html>).

Identification of cell types and features

To identify differentially expressed genes between two cell-type clusters, the Wilcoxon rank-sum test implemented in the *scanpy.tl.rank_genes_groups* function was applied. Genes with an adjusted *p*-value less than 0.05 were considered significantly differentially expressed.

Identification of malignant cells

To rigorously identify malignant cells, we initially isolated putative malignant cell populations via unsupervised clustering. Subsequently, to further eliminate confounding signals from phenotypically similar cells, we implemented logistic regression analysis to exclude bone cells and mesenchymal stromal cells exhibiting transcriptional resemblance. We utilized marker genes (*IGFBP5*, *PTN*, *SERPINF2*, *RUNX2* and *ALPL*) of bone cells as feature genes and trained a logistic regression model to remove potential contaminated cells. Seventy percent of single cells from a normal bone marrow scRNA-seq dataset⁴⁵ (GSE147390) were used as the training set, and the remaining 30% was used as the validation cohort for model optimization. The trained model was then applied to our scRNA-seq dataset, and cells predicted as bone cells were excluded from subsequent analyses. We additionally employed mesenchymal stromal cells-related marker genes (*THY1*, *VIM*, *COL1A1*, *NT5E* and *ENG*) using mesenchymal stromal cells identified

in our dataset (70% for training, 30% for validation) to train a model and further removed cells that were predicted as mesenchymal stromal cells from tumor cells. Subsequently, we performed copy number variation analysis on putative malignant cells, further refining the malignant population by removing non-malignant cells.

CNV analysis

InferCNV (version 1.3.3)¹⁴⁸ (<https://github.com/broadinstitute/inferCNV>) was used to infer copy-number alteration for bone metastatic cancer cells across cancer types. The copy number variations scores of mesenchymal stromal cells, endothelial cells and immune cells were also calculated as a copy number variations control. Then the whole copy number variations profiles were normalized by subtracting average expression profiles of control. Additionally, due to potential confusion between tumor cells and plasma cells in medulloblastoma, we performed CNV calculation on the classified plasma cells to exclude potentially contaminating tumor cells. The scores were restricted to the range -1 to 1 by replacing all values > 1 with 1 and all values < -1 with -1 , and any score between -0.3 and 0.3 was set to 0 . Cells with inconsistent copy number changes were removed and were not included in subsequent analysis of cancer cells.

Non-negative matrix factorization (NMF) analysis

cNMF (version 1.6)¹⁴⁹ (<https://github.com/dylkott/cNMF>) was used to infer gene expression programs of bone metastatic cancer cells. To reduce the impact of high variability in tumor cell numbers among different samples, the number of components (K), which defines the number of co-expressed genes, was set to increase with the malignant cell number in each sample. Samples with a cancer cell count less than 200 were removed from the tumor program analysis. The selection of K-values for different samples was performed using consensus clustering. For each features factor, 50 genes with the highest NMF scores were defined as a signature. All of signatures from 35 samples were combined and did hierarchical clustering using 1 minus Jaccard index as the distance metric to identify recurrent expression programs across human bone metastases. 15 programs were depicted in this study. For each program, the expression score using genes in the program and ranked all the genes by their correlation with the expression score. The top 30 correlated genes were selected to defined programs. Further, Pearson correlation coefficients were calculated between 15 programs for inferring the co-occurrence of programs using signature gene score.

Pathway enrichment analysis

The top-ranked genes identified from differential expression analysis were submitted to the Metascape web tool (version 3.5)¹⁵⁰ (<https://metascape.org/>) for functional enrichment analysis. Default parameters were used, and enriched biological processes or pathways were selected based on statistical significance and biological relevance.

Pathway enrichment network

To visualize functional differences in enriched pathways, we employed the aPEAR¹⁵¹ (version 1.0) R package to construct pathway enrichment networks. Input data were obtained from Metascape-based enrichment analysis and included Description (pathway name), LogP, Log(q-value), Symbols (gene set composition), InTerm_InList (number of overlapping genes/total number of genes in the pathway). The numerator in InTerm_InList was extracted and used as the count variable for node size mapping. Pathways with Log(q-value) < -1.3 were retained for visualization. Network graphs were generated using the enrichmentNetwork function, with colorBy = 'Log.q.value.' and nodeSize = 'count' as the key visualization parameters.

Correlation analysis

Correlation analysis was employed to examine the association between intercellular infiltration patterns and gene expression signatures within the bone metastatic tumor microenvironment. The correlation between EC4 and cancer cells proportion at the pan-bone metastases level was calculated by Pearson correlation. The correlation between 15 programs was calculated by Spearman correlation. The correlation between program signature scores and cell proportion among samples was calculated by Spearman correlation.

Hierarchical clustering

Unsupervised hierarchical clustering was used to analyze the relationship of 15 programs based on program signature gene NMF score, and analyze the relationship of samples based on program signature gene expression. In addition, the feature of myeloid cells and T cells subtypes among cancer types were compared by unsupervised hierarchical clustering, based on feature genes expression. Unsupervised hierarchical clustering analysis was performed using the R packages pheatmap (version 1.0.12) (<https://github.com/raivokolde/pheatmap>) with parameter clustering_method = "ward.D2".

Tissue distribution of cells

To quantify the tissue preference of each cell type or subtype, the ratio of observed to expected cell numbers (Ro/e) was calculated for each cell type or subtype between BM and primary cancers. The Ro/e was calculated as the ratio of observed cell numbers over the expected cell numbers of a given cell type between BM and primary cancers, where the expected cell numbers for each combination of cell types and tissues were obtained from Chi-square test. The absolute count of each cluster was used in this analysis.

The $Ro/e > 1$ represents higher abundance of a certain cell type in bone metastases than primary tumors, whereas $Ro/e < 1$ indicates cell type depleted in bone metastases.

Calculation of signature score

We use `scanpy.tl.score_genes` function¹⁵⁵ to calculate the signature score of a specific gene set. The score is the average expression of a set of genes subtracted with the average expression of a reference set of genes. The reference set is randomly sampled from the `gene_pool` for each binned expression value. To minimize bias introduced by gene expression magnitude variations, the background reference set was constructed using an expression-stratified sampling strategy. This involved partitioning all genome-wide genes into 20 equally sized expression bins based on mean expression levels, followed by random selection of control genes within each bin that parametrically matched the size of the target gene set. This sampling process was repeated 1000 times to generate a robust aggregated background model.

Diffusion component analysis

Diffusion map algorithm was used to identify the major components of variation across MSC subsets. Gene expression normalization was performed using unit variance scaling; the standardization and PCA followed the same methodology as described previously. For the PCA-reduced features of mesenchymal stromal cells, we further computed their diffusion features. Then the first 10 principal components as input of `DiffusionMap` function in `destiny` (version 2.14.0)¹⁵² (<https://github.com/theislab/destiny>). The diffusion patterns of mesenchymal stromal cells subpopulations was visualized using the most probable DC1 and DC2 components.

RNA velocity analysis

ScRNA-seq datasets of bone metastases were used for cell state transition analysis by RNA velocity inference.¹⁵⁶ Future transcriptional states of cells were inferred based on mRNA splicing dynamics. The spliced and unspliced RNA counts of each gene were recounted using `velocity` (version 0.17.17).¹¹⁰ Then RNA velocity was estimated by `scVelo` (version 0.2.4)¹¹⁰ with `scvelo.tl.velocity` function. The stochastic model was selected and `scvelo.tl.velocity_graph` function was used to build velocity graph with default parameters. The RNA velocity was projected into UMAP or diffusion map with `scvelo.pl.velocity_embedding_stream` function for visualization. This pipeline rigorously adheres to the gold standard for single-cell kinetic analysis. All parameters not explicitly specified in the analysis were set to their default values.

CytoTRACE analysis

The raw gene expression profiles were subjected to `cytoTRACE`⁹⁵ (version 3.0.0) analysis, and genes with extremely low expression across all cells were filtered out. For each cell, the expression levels of all genes were sorted in descending order, with the highest-expressed gene assigned the greatest weight and the lowest-expressed (but non-zero) gene assigned the smallest weight, thereby constructing a weighted gene expression profile. Based on this weighted expression profile, a score termed `cytoTRACE` was calculated for each cell. All parameters not explicitly specified in the analysis were set to their default values.

Cell-cell interaction analysis

`CellPhoneDB` (version 2.0.0)⁷⁷ was used to explore the potential interactome between different cell types in BM microenvironment. The potential interaction strength between two cell subsets was predicted based on expression of ligand-receptor pairs. The enriched ligand-receptor interactions between two cell subsets were calculated based on permutation test. Cell-cell interactions were inferred using the `LIANA+` framework,¹⁵⁷ applying the `CellPhoneDB` method on log-normalized data. The consensus ligand-receptor resource was used (`resource_name = 'consensus'`), and only interactions with both ligand and receptor expressed in at least 10% of cells within each cluster were considered (`expr_prop = 0.1`).

TF analysis

Activated TFs regulons in each CD8-Tex subsets were analyzed using `SCENIC`.¹⁵³ Raw count matrix was used as input for `pySCENIC` (version 0.11.2). `GRNBoost` (Gradient Boosting) infers co-expression modules between transcription factors and candidate target genes, with each module comprising a transcription factor and its target genes. `RcisTarget` was used to analyze genes within each co-expression module to identify enriched motifs, retaining only those modules and targets exhibiting TF motif enrichment. This constructs a TF-targets network where each transcription factor and its potential direct target genes are defined as a regulon. Finally, using `AUCell` to quantify the activity of validated regulons. The activated transcription factors were inferred for each subpopulation of CD8 exhausted T cells.

Survival analysis

The clinical information of bone metastatic patients was collated. Patients were classified into high and low groups based on the specific cells type proportion or gene expression. Which described in figure legends. Two grouping strategies were employed: (1) median-based classification, where patients were divided into high and low groups according to the median value; and (2) data-driven optimal cutoffs determined using the `surv_cutpoint()` function from the `survminer` package, which identifies the value that best separates survival outcomes. Survival curves were fit using the Kaplan-Meier formula in `survival` (version 3.2.11) (<https://github.com/>

[therneau/survival](#)), and displayed using the *ggsurvplot* function of *survminer* (version 0.4.9) (<https://github.com/kassambara/survminer>).

Mass cytometry data analysis

Each sample's data was barcode-removed from raw data using a bimodal filtering scheme with unique quality-labelled barcodes.¹⁵⁸ Normalisation of each.fcs file generated from different batches by the bead normalization method.¹⁵⁹ Debris, dead cells and doublets were excluded from gate data using FlowJo software. Live, single immune cells were leaved for analysis. The Leiden clustering algorithm was applied to cells to classify them into different phenotypes based on marker protein expression levels. The high-dimensional data were visualised in two dimensions using the dimensionality reduction algorithm UMAP, and the distribution of each cluster and marker expression was shown, as well as the differences between each group or different sample types. Markers of T cell subtypes used in mass cytometry datasets were collected in [Table S3](#).

MET500 data analysis

Bone metastases samples RNAseq data from MET500 datasets⁵⁶ was downloaded. The 15 programs were defined in bone metastases samples of MET500 by program signature genes expression. The correlation of programs finding in bone metastases samples of MET500 datasets was analyzed. And the relationship of these samples was assessed by unsupervised hierarchical clustering based on program signature gene expression.

Statistical analysis

Kruskal-Wallis test, Wilcoxon test, Student's *t* test and one-way ANOVA were used in this study, and described in figure legends. TheKaplan-Meier method was applied in survival analyses. All of the statistical details of experiments, including the statistical tests used, exact value of n et al. can be found in the figure legends.