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Breast cancer–osteoclast interactions generate hybrid-like cells via non-canonical fusion and cell-in-cell processes

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Abstract

Background: Bone-metastatic breast cancer cells adapt to the skeletal microenvironment through osteomimicry, whereas macrophage-lineage osteoclasts are highly fusogenic cells that directly interact with disseminated tumor cells. However, the mechanisms underlying the interactions between breast cancer cells and osteoclasts in bone remain poorly defined.

Methods: Murine breast cancer cells were co-cultured with RAW264.7-derived or primary bone marrow-derived osteoclasts under osteoclastogenic conditions. Their interactions were examined using fluorescence-based imaging, Cre-Lox reporter systems, molecular analyses, cytokine profiling, and laser microdissection followed by SMART-seq2. Bone metastasis models were established by caudal artery injection of reporter-labeled breast cancer cells, and flow cytometry was used to identify tumor cells with Cre-Lox reporter activation following close interaction with osteoclasts in vivo. Public single-cell RNA-sequencing data from human breast cancer bone metastases were analyzed to establish the patient-level relevance of the identified hybrid-like cell state.

Results: Breast cancer cells dynamically interacted with developing osteoclasts through fusion and cell-in-cell-like processes, generating osteoclast-tumor hybrid-like cells. These cells exhibited distinct extracellular matrix-remodeling, angiogenic, and cancer-associated transcriptional programs, including PI3K-AKT and Hippo/YAP signaling. Conversely, osteoclast

interactions induced hematopoietic and osteoclast-lineage features in breast cancer cells, with stronger reprogramming under direct-contact conditions. Cre-Lox reporter activation identified tumor cells that had interacted with osteoclast-lineage cells in murine bone metastases. Single-cell RNA-sequencing analysis further supported the presence of comparable hybrid-like states in human breast cancer bone metastases.

Conclusions: Direct interactions between breast cancer cells and osteoclasts generate hybrid-like cell states with distinct matrix-remodeling, immunoregulatory, and cancer-associated programs. These reciprocal interactions may represent a previously unrecognized mechanism of tumor adaptation within the bone metastatic niche.

Keywords

Breast cancer bone metastasis; Osteoclast–tumor hybrid-like cells; Cell fusion; Cell-in-cell; Osteomimicry

Background

Cell fusion is a source for tumor heterogeneity in cancer [1, 2], and heterotypic fusion between tumor and stromal cells can generate aggressive, invasive tumor variants [3, 4]. CIC formation, including entosis, emperipolesis, cannibalism, and phagocytosis, is another cell–cell interaction linked to tumor progression, immune evasion, drug resistance, and aneuploidy[5, 6]. Nevertheless, comprehension on cell fusion and CIC processes and how they influence cancer cell fate remains poorly understood.

Osteoclasts are multinucleated myeloid-derived cells generated through precursor fusion. They are central mediators of tumor-induced osteolysis in bone metastasis [7]. In osteolytic lesions, tumor–osteoclast crosstalk through secreted factors and extracellular vesicles drives a vicious cycle of bone resorption and tumor growth [8, 9]. Recent evidence showed that direct cell-cell contact between osteoclasts and tumors can hasten osteoclast maturation[10], and osteoclasts containing tumor cells within their cytoplasm have been reported[11]. These

observations suggest that direct physical interactions between tumor cells and osteoclasts may contribute to bone metastatic progression.

Evidence for osteoclast–tumor fusion has also been reported in myeloma bone metastasis, where osteoclasts contained B-cell-derived nuclei carrying myeloma-specific chromosomal translocations [12]. Given that many solid tumors display strong bone tropism, we hypothesize that disseminated tumors may similarly interact with osteoclasts through cell fusion/CIC routes to form osteoclast-tumor hybrid-like cells.

Here, we show that a rare subset of breast cancer cells in human bone metastasis express osteoclast-associated matrix-remodeling and osteomimicry signatures. Using live imaging, laser microdissection, Smart-seq2 profiling, in vivo lineage-tracing models, and patient single-cell data, we identify dynamic fusion/CIC-like interactions between tumor cells and developing osteoclasts. The resulting hybrid-like cells retain transcriptionally active tumor nuclei and acquire ECM-remodeling, angiogenic, immunoregulatory, and cancer-associated pathway activity, including PI3K–AKT and Hippo/YAP signaling. These findings reveal osteoclast–tumor hybrid-like cells as a distinct cell state in bone metastatic lesions and suggest a direct-contact mechanism by which tumor cells remodel osteoclast biology.

Methodology

Cell Culture

RAW264.7, 4T1, EO771, B16F10, and CT26 cells were obtained from ATCC and cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO₂. Primary bone marrow cells were isolated from C57BL/6 mice. Bone marrow-derived macrophages (BMMs) were generated with 25 ng/ml mM-CSF for 3 days, followed by osteoclast differentiation using 10 ng/ml mRANKL, 25 ng/ml mM-CSF, and 40 ng/ml mTNF- α for 3 days. RAW264.7 cells were differentiated with 100 ng/ml mRANKL, 75 ng/ml mM-CSF, and 40 ng/ml mTNF- α for 5 days, with medium replacement on day 2.

Lentiviral transduction

RAW264.7 macrophages were transduced with lentivirus generated from pLenti-CMV-GFP-Hygro to produce RAW264.7-GFP cells after hygromycin selection. 4T1, EO771, B16F10, and

CT26 cells were transduced with pHIV-H2B-mRFP lentivirus to generate H2B-mRFP cancer cell lines, followed by sorting using a Sony SH800Z cell sorter. EO771 and B16F10 Cre-reporter lines were generated using lentivirus carrying a floxed DsRed-E2 cassette with downstream eGFP, followed by puromycin selection. Plasmids were purchased from Addgene: pLenti-CMV-GFP-Hygro (656-4, Addgene #17446), pHIV-H2BmRFP (Addgene #18982), and Cre Reporter (Addgene #62732).

Osteoclast precursor-cancer cells co-cultures

RAW264.7-GFP and 4T1-H2B-mRFP cells were seeded at a 20:1 ratio on day 0 with 100 ng/ml mRANKL, 75 ng/ml mM-CSF, and 40 ng/ml mTNF- α . Medium containing the same cytokines was replaced on day 2, and cells were co-cultured up to day 5. For other cancer cell lines, seeding density was adjusted according to doubling time. Differentiated primary BMMs were mixed with EO771 Cre-reporter cells at a 37:1 ratio and cultured with primary osteoclast differentiation cytokines for up to 3 days. For downstream assays, RAW264.7/4T1 co-cultures were magnetically sorted twice using mouse CD45 MicroBeads (Miltenyi, 130-052-301) to obtain CD45⁺ RAW264.7 osteoclast and CD45⁻ 4T1 tumor fractions.

Western Blot

Proteins were extracted, quantified by BCA assay, separated by SDS-PAGE, and transferred to membranes by wet transfer. Membranes were blocked with 1% BSA and probed with anti-PU.1/Spi1 antibody (31 kDa; rabbit recombinant monoclonal, clone EPR22624-20, Abcam, ab227835; 1:1000) and anti- β -actin antibody (42 kDa; mouse monoclonal, clone AC-15, Sigma-Aldrich, A5441; 1:5000). Detection was performed using species-appropriate HRP-conjugated secondary antibodies and Clarity Western ECL Substrate (Bio-Rad, #1705061).

RT-qPCR

RNA was isolated using FastPure Cell/Tissue Total RNA Isolation Kit V2 (#RC112) and reverse-transcribed using PrimeScript RT Master Mix Kit (Takara Bio, Inc.). qPCR was performed using PowerUp[™] SYBR[™] Green Mix (Applied Biosystems[™], A25742). β -actin was used for normalization, and relative gene expression was calculated using the 2^{- $\Delta\Delta$ Ct} method. Primer sequences are listed in Supp. Table 1.

Immunocytochemistry

Cells were fixed with 4% PFA, permeabilized with 0.1% Triton-X, and blocked with 1% BSA and 300 μ M glycine in PBS containing 0.1% Tween-20 for 30 min. Primary antibodies were incubated overnight at 4 °C, followed by secondary antibody staining for 1 h at room temperature. Antibodies used were anti-RFP (Rockland, 200-101-379), anti-H3K4me2 (Invitrogen, PA5-114685), anti-GFP (Aveslabs, GFP-1020), Yap (D8H1X) XP Rabbit mAb (CST, 14074S), anti-p21 antibody (Abcam, ab188224), AlexaFluor488 AffiniPure™ donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch, 711-545-152), AlexaFluor Plus 647 donkey anti-goat IgG (H+L) (Invitrogen, A32849), AlexaFluor Plus 405 Phalloidin (Invitrogen, A30104), and Alexa Fluor 488 AffiniPure donkey anti-chicken IgY/IgG (H+L) (Jackson ImmunoResearch, 703-545-155). Images were acquired using a Nikon A1HD25 Confocal Microscope with Nikon NIS Elements and processed using ImageJ/Fiji.

Time-lapse live cell recording

Cell fusion dynamics were monitored using the CytoSMART™ Lux3 FL fluorescence live-cell imager. Brightfield and green/red fluorescence images were recorded from day 0 to day 5, with fields adjusted to track cells of interest. Snapshot images and time-lapse recordings were processed using the Axion Biosystems online server system.

RAW264.7-GFP-4T1-H2B-mRFP co-culture on laser microdissection dish

RAW264.7-GFP and 4T1-H2B-mRFP cells were co-cultured on polyethylene naphthalate (PEN) membrane dishes for laser microdissection. PEN Petri Dishes 4 μ m (11600295) were obtained from Leica. Cells were seeded evenly, and osteoclastogenic medium was added dropwise to minimize disruption. To prevent dehydration, dishes were placed in a 10 cm² dish with peripheral PBS. Medium was replaced on days 2 and 4, and cells were microdissected on day 5.

Laser microdissection

Cells were isolated using a Leica LMD7000 microscope based on fluorescence. Osteoclast–tumor hybrid-like cells were defined as GFP⁺ osteoclasts containing intact mRFP⁺ tumor nuclei; non-hybrid osteoclasts were GFP⁺ osteoclasts without mRFP⁺ tumor material; tumor cells were mRFP⁺ single cells. Excised cells were collected into lysis buffer containing 2.5 mM dNTP mix (Invitrogen, R0192), 1 U/ μ l SUPERase-In™ RNase Inhibitor (Invitrogen, AM2694), and 0.1%

144 Triton™ X-100 (Sigma, 9036-19-5) in RNase-free ddH₂O, then frozen on dry ice and stored at
145 -80 °C.

146 **Cytokine array**

147 Co-culture supernatants were analyzed using the Proteome Profiler Mouse XL Cytokine Array
148 (Biotechne). Membranes were imaged using the ChemiDoc Imaging System (Bio-Rad), and
149 spot intensity was quantified using Image Lab software (Bio-Rad).

150 **Mouse experiment**

151 All animal experiments were approved by the Animal Experimentation Ethics Committee of
152 City University of Hong Kong (Ref. No.: AN-STA-00000207). Mice were housed at 24 ± 1 °C
153 under a 12 h dark-light cycle with standard chow. Ctsk-2A-Cre mice (Cat. No. NM-KI-190019)
154 were purchased from Shanghai Model Organisms Center, Inc., and WT C57BL/6 mice were
155 provided by the Laboratory Animal Research Unit of City University of Hong Kong (ISO 9001
156 verified). Mice aged 8–12 weeks were used. Male mice were used for B16F10 and female mice
157 for EO771 bone metastasis models. Ctsk-Cre genotyping primers were: Ctsk-cre (Mut),
158 GAGAGCTGGGGAAACAAAG and GGTAGTCCCTCACATCCTCAG; Ctsk-cre (Wt),
159 TCTACCTTCAAAGTGCTGCCATTA and AGAGAAGGGAAGTAGAGTTGTCAC.

160 **Bone metastasis model via caudal artery injection**

161 Bone metastasis was induced as described by Kuchimaru, T., et al. [13]. Mice were sedated
162 with pentobarbital sodium by intraperitoneal injection, and 250,000 cells in 100 µl were
163 injected through the caudal artery. Bone metastatic development was monitored using an
164 IVIS® Lumina Series III system. Mice were shaved before imaging to reduce autofluorescence.
165 DsRed fluorescence from EO771 and B16F10 Cre-reporter cells was detected, and
166 background/autofluorescence was removed by spectral unmixing. Each tumor group included
167 ≥10 biological replicates.

168 **Tumor and bone marrow processing in mice**

169 Mice were sacrificed upon signs of pain or distress. Bone metastases were collected and
170 dissociated into single cells using the Singleron PythoN® Tissue Dissociation System. BM cells
171 from metastatic hind limbs were flushed and collected as single-cell suspensions. Bone
172 metastasis and BM analyses used ≥4 and ≥3 biological replicates, respectively.

Flow cytometry

Cells were blocked with TruStain FcX™ anti-mouse CD16/32 antibody (Biolegend, 101320). Live cells were assessed directly for DsRed and GFP fluorescence. Fixed cells were processed using BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences, 554714) before staining for GFP and DsRed fluorescent proteins. Anti-RFP antibody (Rockland, 200-101-379) was used to stain DsRed when required. Data are shown as mean ± SD. Statistical significance was evaluated using two-tailed unpaired Mann–Whitney U test unless otherwise indicated. $P < 0.05$ was considered significant. Figures were generated using GraphPad Prism.

SMART-seq 2 single cell transcriptome sequencing

Frozen single-cell RNA samples were submitted to BGI Genomics for quality control and SMART-seq2 sequencing. Each cell type included ≥5 biological replicates. Sequencing was performed on the DNBSEQ platform using a PE100 full-transcriptome strategy.

RNA sequencing data preprocessing

Raw reads were quality-controlled using FastQC [14], aligned to the *Mus musculus* genome (NCBI:GCA_000001635.8) using STAR [15], and quantified using RSEM [16]. PCA was used to assess batch effects and sample distribution. DEGs were identified using DESeq2 [17]. Gene Ontology and KEGG enrichment analyses were performed using clusterProfiler [18], and results were visualized with ggplot2 [19].

Public Database Comparison

Single-cell RNA-seq data from ER⁺ breast cancer bone metastasis (GSE190772) were used [20]. Osteoclast and epithelial tumor populations were extracted, normalized using Seurat v5.1.0 [21], and visualized by UMAP. Human gene annotations were matched with *Mus musculus* genes for cross-species analysis.

GSVA v1.52.3 [22] was used to compare DEGs from experimental datasets with individual osteoclasts from the public dataset. Additional GSVA was performed using KEGG pathways of interest.

Results

Rare breast cancer subpopulation in bone metastases exhibit osteoclast-associated matrix-remodeling and osteomimicry signatures

Breast cancer cells with strong bone tropism have been described to adopt bone-like osteomimicry [23, 24]. To investigate whether breast cancer cells display osteoclast-like features, we analysed a publicly available single-cell sequencing dataset derived from ER+ breast cancer bone metastases (GSE190772). Epithelial and osteoclast populations were visualized using the original cluster annotations (Fig. 1a). We curated an osteoclast/osteomimicry (OC/OM) gene panel encompassing osteoclast-associated resorptive/matrix-remodeling enzyme genes (*Ctsk*, *Acp5*, *Ca2*, *Mmp2*, *Mmp9*, *Mmp14*) and osteomimicry-associated genes (*Spp1*, *Ibsp*) and compared their expression between epithelial and osteoclast clusters (Fig. 1b). OC/OM genes were mainly enriched in osteoclasts, whereas *Ibsp* showed broader expression patterns. Notably, OC/OM module scoring revealed a small tail of epithelial cells with scores comparable to osteoclasts, suggesting a rare epithelial subset with osteoclast-associated and osteomimicry features (Fig. 1c).

We next quantified the number of OC/OM genes detected per cell and found that a minor epithelial fraction expressed five or more OC/OM genes at levels comparable to osteoclasts (Fig. 1d). These findings indicate that rare breast cancer cells in bone metastases acquire OC/OM-associated gene expression. We then examined the five epithelial subclusters (1, 3, 4, 5, 6) (Fig. 1e). UMAP visualization showed that subclusters 1, 3, and 6 displayed broad but low-level OC/OM expression, whereas the strongest signals were concentrated in small fractions of subclusters 4 and 5 (Fig. 1f). Intensity-versus-prevalence analysis confirmed this high-intensity, low-prevalence pattern in subclusters 4 and 5 (Fig. S1a).

Distributional analysis further showed that OC/OM log1p raw counts in subclusters 1, 3, and 6 were largely restricted below 0.5, whereas subclusters 4 and 5 contained a distinct high-expression tail exceeding 1.0 (Fig. 1g). Amongst epithelial cells with positive OC/OM-expression, subclusters 4 and 5 showed normalized values of 2–4, compared with 0–2 in subclusters 1, 3, and 6 (Fig. 1h). Collectively, these results identify a rare OC/OM-like epithelial subset in bone metastasis that expresses osteoclast-associated osteomimicry patterns.

Breast cancer cells undergo dynamic cell fusion and CIC structure formation with developing osteoclasts

Metastasized cancer cells can physically interact with osteoclasts in the bone niche[25]. Given the OC/OM-like features observed in breast epithelial tumor cells, we tested whether breast cancer cells can integrate into developing osteoclasts. RAW264.7-GFP osteoclast precursors were co-cultured with 4T1-H2B-mRFP breast cancer cells (Fig. 2a). RFP⁺ tumor nuclei were detected within mature multinucleated RAW264.7-GFP osteoclasts under osteoclastogenic conditions, but not without osteoclastogenic cytokines (Fig. 2b; data not shown). Similar tumor-nucleus inclusion was observed in primary bone-marrow-derived osteoclasts co-cultured with 4T1-H2B-mRFP cells (Fig. 2c).

Live-cell imaging showed that tumor–osteoclast integration occurred dynamically during osteoclast differentiation. Tumor cells entered developing osteoclasts during pre-osteoclast fusion (Fig. 2d, Movie S1), and multinucleated hybrid-like osteoclasts containing tumor nuclei could further fuse with each other (Fig. 2e, Movie S2). Individual tumor cells and pre-osteoclasts also entered pre-existing hybrid-like osteoclasts, with incorporated nuclei converging toward the central nuclear region (Fig. 2f, Movie S3). In our observation, most tumor nuclei get incorporated into the host osteoclast nuclei after entry. Similar events were observed in primary BM-derived osteoclasts co-cultured with EO771-DsRed-E2 (Fig. S2a,b, Movie S4), as well as with B16F10 melanoma and CT26 colon cancer cells, suggesting that tumor–osteoclast integration is not breast cancer-specific (Fig. S3a,b).

Further imaging showed that incorporated tumor nuclei retained surrounding membrane structures, whereas osteoclast nuclei either retained membranes or merged into the shared cytoplasm (Fig. 2g). Both cell types adopted a rounded morphology consistent with pre-osteoclast fusion behavior prior to fusion [26], which suggest combined CIC-like entry and canonical osteoclast fusion.

To assess tumor-nucleus activity residing within osteoclasts, we stained for H3K4me₂, a marker of actively transcribed loci [27]. Many RFP⁺ tumor nuclei colocalized with H3K4me₂, whereas fragmented H3K4me₂-negative tumor nuclei were also observed within the same hybrid-like cells (Fig. 2h), suggesting heterogeneous transcriptional states among incorporated tumor nuclei. Live tracking further captured tumor nuclear shrinkage and

disintegration adjacent to osteoclast nuclei, resembling an entosis-like fate in CIC formation for an isolated case (Fig. S4a, Movie S5). Together, these findings indicate that breast cancer cells enter osteoclasts through dynamic osteoclast fusion and CIC-like processes, generating heterogeneous osteoclast–tumor hybrid-like cells.

Osteoclast-tumor hybrid-like cells acquire ECM-remodeling and cancer-associated transcriptional programs

To define osteoclast–tumor hybrid-like cells, we laser-microdissected hybrid-like osteoclasts containing tumor nuclei (OHC), non-hybrid osteoclasts from co-cultures (OC), and osteoclasts from osteoclast-only cultures (O) based on fluorescent protein expression (Fig. 3a). Representative cells were imaged before isolation (Fig. 3b), and RNA-quality-passed samples were subjected to Smart-seq2 sequencing (Fig. S5a,b). Differential expression analysis showed that OHCs had approximately twice as many DEGs as OC when compared with O, and nearly 200 DEGs separated OHC from OC (Fig. 3c). DEG heatmaps highlighted OHC-specific expression patterns, whereas OC and O were more similar (Fig. 3d).

DEG overlap analysis revealed 498 and 215 non-overlapping DEGs in OHC vs. O and OC vs. O, respectively, with most OHC vs. OC DEGs also present in OHC vs. O (Fig. 3e). PCA showed close clustering of OC and O, whereas OHCs were more dispersed, consistent with a heterogeneous but distinct osteoclast state (Fig. 3f).

GO analysis showed that OHC vs. O was enriched for angiogenesis, ECM remodeling, cell motility, epithelial/mesenchymal transformation, focal adhesion, and cell–cell junction programs, whereas OC vs. O was dominated by inflammatory and cytokine-related responses (Fig. 3g). OHC vs. OC similarly enriched ECM organization, basement membrane assembly, tight junction formation, Hippo signaling, and angiogenesis (Fig. 3h), while OHC vs. O showed reduced bone mineralization, ossification, and myeloid differentiation programs (Fig. 3i).

KEGG analysis for OHC vs. O showed enriched PI3K–Akt, Hippo, ECM–receptor interaction, focal adhesion, junctional, cytokine/chemokine, complement, xenobiotic, and glutathione metabolism pathways (Fig. 3j). In contrast, OC vs. O enriched IL-17, chemokine, C-type lectin receptor, NF- κ B/MAPK/TNF signaling, hematopoietic lineage, and osteoclast differentiation

pathways (Fig. 3k). OHC vs. OC confirmed stronger cancer-associated, ECM-remodeling, PI3K–Akt, and Hippo signaling in OHCs (Fig. 3l), and KEGG network analysis summarized this divergence between cancer/adhesion-associated OHC programs and immune/inflammatory OC programs (Fig. 3m).

Osteoclasts reprogram breast cancer cells toward hematopoietic and osteoclast-like states

We next examined how osteoclasts altered tumor-cell states. Tumor cells from co-cultures (T_CO) were isolated by laser microdissection for single-cell sequencing, with osteoclastogenic cytokine-treated tumor cells (T_T) and untreated tumor cells (T_UT) as controls (Fig. 4a). T_CO cells were collected from tumor-only clusters to avoid osteoclast contamination, and RNA quality was confirmed before sequencing (Fig. S6a,b).

Differential expression analysis showed extensive transcriptional changes in T_CO compared with both controls, whereas T_T and T_UT differed minimally (Fig. 4b). DEG heatmaps confirmed a distinct T_CO profile, while T_T and T_UT remained similar (Fig. 4c). T_CO vs. T_UT and T_CO vs. T_T shared approximately half of their DEGs, whereas T_T vs. T_UT showed little overlap with either comparison (Fig. 4d). PCA further separated T_CO from the two control groups (Fig. 4e), indicating that osteoclast presence, rather than osteoclastogenic cytokines alone, drives tumor-cell reprogramming.

GO analysis of upregulated DEGs showed enrichment of hematopoietic differentiation, antigen processing, extracellular/membrane-associated functions, and ligand–receptor activity in T_CO cells (Fig. 4f). Osteoclast fusion and differentiation genes, including *Dcstamp*, *Ocstamp*, *Oscar*, and *Trem2*, were also upregulated (Fig. S7a,b). KEGG analysis similarly enriched immune-response pathways, osteoclast differentiation, phagosome/lysosome regulation, PI3K–AKT signaling, and glycolysis-related metabolism, while protein translation-associated functions were attenuated in T_CO vs. T_T (Fig. 4g; Fig. S8a,b).

Across all tumor and osteoclast populations, PCA showed clear separation between tumor cells and osteoclasts, with OHCs aligning more closely with osteoclasts than tumor cells (Fig. 4h). KEGG network analysis showed cancer-related and cytokine pathways in OHCs, stronger

metabolic and hematopoietic pathway activation in T_CO cells, and shared IL-17, TNF, and RA signaling between both states (Fig. 4i).

Direct spatial contact with osteoclasts influences breast cancer cell behaviour and microenvironment

Tumor cells can spatially interact with bone-lineage cells in metastatic niches [25, 28, 29]. To test whether proximity influences tumor adaptation, we compared direct and transwell tumor–osteoclast co-cultures (Fig. 5a). Cytokine arrays showed that direct contact increased immunosuppressive, pro-angiogenic, and ECM-remodeling factors, including CCL22, IL-10, VEGFA, PDGFb, MMP2, MMP9, and PTX3, whereas transwell co-culture enriched inflammatory mediators such as CSF2, IL-23, and CXCL16 (Fig. 5b). Thus, direct tumor–osteoclast contact shifted the secretory environment toward angiogenic, immunoregulatory, and matrix-remodeling programs.

We next assessed whether direct contact induced osteoclast-like reprogramming in tumor cells. CD45-based magnetic separation yielded >99% pure osteoclast and breast cancer fractions by flow cytometry (data not shown). qPCR showed strong induction of osteoclast/hematopoietic genes in directly co-cultured tumor cells, with minimal changes under transwell conditions (Fig. 5c). *Spi1* showed the strongest induction, reaching ~14 log₂ fold increase, while *Fcer1g*, *Dap12*, *Dcstamp*, *Ocstamp*, *Trem2*, *Car2*, and *Atp6v0d2* were also elevated. OC/OM panel genes, including *Car2*, *Ctsk*, *Acp5*, *Mmp9*, and *Mmp2*, were similarly upregulated. It is notable that not all genes exhibited higher expression levels under direct contact as *Cd74*, *Fos*, and *Mmp9* were equally expressed in both conditions. Dorothea analysis identified *Spi1*, together with *Nfkb1*, *Rela*, and *Hif1a*, as activated transcription factors in TUMOR_CO relative to TUMOR_UT (Fig. 5d). Western blotting confirmed *Spi1* protein expression in breast cancer cells only under direct-contact conditions, not in transwell or tumor-only controls (Fig. 5e).

To validate osteoclast–tumor association in vivo, EO771 breast cancer or B16F10 melanoma cells carrying a lox-DsRed-STOP-lox-GFP Cre reporter were injected into *Ctsk*-Cre mice by caudal artery (Fig. 5f), and DsRed fluorescence was used to monitor bone metastasis (Fig. 5g). Flow cytometry detected GFP⁺ recombined tumor cells in bone metastatic lesions and bone

marrow from Ctsk-Cre mice, but not wild-type controls, in both tumor models (Fig. 5h,i; Fig. S9a). GFP⁺ cells were absent from extraskeletal metastases, including liver, spleen, lung, and abdominal lesions (Fig. 5j). Collectively, these findings indicate that direct osteoclast association reshapes the metastatic microenvironment and induces osteoclast-like tumor-cell reprogramming.

Osteoclast-tumor hybrid-like cells are found in human breast cancer-bone metastasis

To assess whether osteoclast–tumor hybrid-like states occur in patients, we analyzed the ER⁺ breast cancer bone metastasis dataset [20]. Focusing on annotated osteoclasts (Fig. 6a), we applied GSVA using OHC vs. O and OC vs. O DEG signatures (Fig. 6b). High OHC-scoring cells formed a discrete OHC-like cluster, whereas OC-like cells were distributed across two partially overlapping regions, consistent with the partial but distinct relationship between OHC and OC observed in our Smart-seq2 data.

Pathway-level GSVA further separated these states (Fig. 6c,d). OHC-associated pathways, including ECM–receptor interaction, Hippo signaling, and PI3K–AKT signaling, were concentrated within the isolated OHC-like cluster, whereas OC-associated inflammatory and osteoclast-related pathways, including C-type lectin receptor signaling, osteoclast differentiation, and lipid/atherosclerosis pathways, were enriched mainly in OC-like regions in the main body cluster. Functional annotation showed that the OHC-like cluster shared key programs with experimentally isolated hybrid-like cells (Fig. 6e), and quantitative pathway-profile comparison confirmed significantly higher similarity to OHC than OC profiles (Fig. 6f). Overall, these findings support the presence of OHC-like osteoclast states in human breast cancer bone metastasis.

Hippo “off” pathway in osteoclast-tumor hybrid-like cells

Transcription factor analysis showed stronger *Tead1* activity and *Yap1/Taz* expression in OHCs than OCs, consistent with Hippo pathway inactivation (Fig. 7a). In CD45⁺ osteoclast-lineage cells from direct versus paracrine co-cultures, direct contact increased ECM-associated genes (*Fn1*, *Pcolce2*, *Col4a2*) and Hippo “off”/YAP target genes (*Yap1*, *Amotl2*, *Ankrd1*, *Areg*, *Ctgf*,

Cyr61, *Itga2*, and *Serpine2*) without markedly altering classical osteoclast enzymes (Fig. 7b). This implies the activation of ECM-remodeling and YAP-associated programs in direct-contact osteoclast populations.

YAP1 staining in primary BM- and RAW264.7-derived co-cultures showed strong YAP1 signals mainly in tumor nuclei within hybrid-like cells and individual tumor cells, whereas osteoclast nuclei showed weak or minimal signal (Fig. 7c). YAP1⁺ tumor nuclei were frequently positioned adjacent to YAP1-low osteoclast nuclei within hybrid-like cells (Fig. 7d). This supports the contribution of incorporated tumor nuclei to the hybrid-like cell state.

YAP1 activity is associated with reduced p21 expression[30]. We thus quantified p21 status in tumor nuclei within OHCs (Fig. 7e). p21-negative tumor nuclei were enriched at the OHC level ($p = 0.00632$), whereas pooled nucleus-level analysis showed 50/102 p21-negative nuclei (49.0%; $p = 0.617$) (Fig. 7f). Thus, individual OHCs frequently contain p21-low tumor nuclei, consistent with a proliferation-permissive hybrid-like state.

Discussion

The role of osteoclasts and myeloid-lineage cells in bone metastasis has largely been framed around paracrine interactions that drive tumor growth and osteolysis [31]. However, emerging evidence that myeloid cells can fuse with tumor cells suggests that direct physical interactions may also influence metastatic progression [1, 32, 33]. Here, we show that solid tumor cells can interact with osteoclasts through dynamic fusion and CIC-like processes during osteoclast differentiation to generate osteoclast–tumor hybrid-like cells. The presence of transcriptionally active tumor nuclei in these hybrids parallels prior observations in myeloma bone metastasis, where osteoclasts contained B-cell-derived tumor nuclei carrying myeloma-specific chromosomal translocations [12]. Our findings extend this concept beyond hematological malignancy and are consistent with reports of osteoclasts harboring tumor cells in thyroid cancer [11]. Although cell-in-cell models have been lacking in osteoclast studies, we surmise that osteoclasts containing solid tumors within their cytoplasm are formed through dynamic combination of cell fusion and cell-in-cell phenomenon. Further in-depth cellular

dynamics studies are required to fully comprehend how both processes were regulated to form hybrid-like cells.

Osteoclasts exhibit diversified functions under inflammatory and disease conditions [34]. Transcriptomic profiling indicated that osteoclast–tumor hybrid-like cells represent a distinct osteoclast state enriched for pro-angiogenic and ECM-remodeling programs. These functions may be relevant to bone metastasis because angiogenesis supports tumor vascularization[35], while ECM deregulation promotes disorganized bone matrices and tumor invasion [36]. Together, these changes may alter mechanotransduction and matrix stiffness to support tumor survival [37]. Hybrid-like cells also activated PI3K–AKT and Hippo/YAP-associated programs. PI3K–AKT signaling supports both cancer progression and osteoclast survival [38], whereas YAP1/TAZ–TEAD signaling regulates proliferation, EMT, and chemoresistance [39]. Consistent with this, the enrichment of p21-negative tumor nuclei in hybrid-like cells suggests a more proliferation-permissive state. However, our study cannot determine whether these hybrids are oncogenic.

We also found that osteoclasts induced breast cancer cells to express hematopoietic and osteoclast-lineage genes. Such lineage plasticity resembles other tumor transdifferentiation programs, including neuron-like differentiation in glioblastoma and osteomimicry in prostate cancer [40], as well as endothelial-like vascular mimicry in several cancers [41, 42]. Notably, circulating tumor cells from patients with bone metastases have been reported to express osteoclast-associated genes [43], raising the possibility that prior interactions with osteoclast-lineage cells contribute to these phenotypes. Collectively, we propose that breast cancer cells undergo a process similar to osteomimicry, which alters their morphology into a detached and round state, similarly observed in pre-osteoclasts prior to osteoclast fusion [44].

Our data further indicate that direct tumor–osteoclast contact is important not only for fusion/CIC-like entry, but also for tumor and osteoclast reprogramming. This aligns with recent evidence that tumor cells can transfer material to osteoclast precursors via direct contact-mediated migrasomes to promote osteoclast differentiation [25]. We acknowledge that tumor cells in our mice study may also acquire Cre activity through similar mechanisms. Nonetheless, the absence of recombined tumor cells outside bone lesions supports a bone-niche-specific interaction with Ctsk⁺ osteoclast-lineage cells.

Finally, direct contact appeared to shift osteoclast-lineage cells away from a purely osteolytic program toward an ECM-remodeling and immunoregulatory phenotype. Although breast tumors are known to exacerbate osteolysis by activating osteoclasts [45], our findings suggest that tumor direct contact with osteoclasts may generate tumor-educated osteoclast states reminiscent of tumor-associated macrophage (TAM) reprogramming.

In conclusion, our study unveiled that breast tumors participate in osteoclast fusion and/or CIC-like processes to generate osteoclast-tumor hybrid-like cells. These hybrid-like cells exhibit immunoregulatory, pro-angiogenic, and ECM-remodelling functions and express cancer-associated PI3K-AKT and Hippo pathways. In bone metastases, this mechanism provides additional immune evasion pathways where breast tumors can camouflage themselves to resemble osteoclasts and by modifying osteoclast phenotype in a similar manner to TAMs. These osteoclast–tumor hybrid-like states may contribute to metastatic bone lesion development and represent a direct-contact mechanism of tumor–bone niche adaptation.

List of abbreviations:

ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
BM	Bone marrow
BMM	Bone marrow-derived macrophage
BP	Biological process
BSA	Bovine serum albumin
CC	Cellular component
cDNA	Complementary DNA
CIC	Cell-in-cell
CMV	Cytomegalovirus
Cre-Lox	Cre-Lox recombination system
DEG	Differentially expressed gene
DNBSEQ	DNA nanoball sequencing
ECM	Extracellular matrix

463 ECL Enhanced chemiluminescence

464 ER+ Estrogen receptor-positive

465 FBS Fetal bovine serum

466 FSC-A Forward scatter area

467 FSC-H Forward scatter height

468 FSC-W Forward scatter width

469 GFP Green fluorescent protein

470 GO Gene Ontology

471 GSVA Gene set variation analysis

472 H2B Histone H2B

473 H3K4me2 Histone H3 lysine 4 dimethylation

474 HRP Horseradish peroxidase

475 IgG Immunoglobulin G

476 IgY Immunoglobulin Y

477 IVIS In vivo imaging system

478 KEGG Kyoto Encyclopedia of Genes and Genomes

479 LMD Laser microdissection

480 MF Molecular function

481 O osteoclast-only cultures

482 OC Non-hybrid osteoclast from co-culture

483 OC/OM Osteoclast/osteomimicry

484 OHC Osteoclast-tumor hybrid-like cell

485 PCA Principal component analysis

486 PBS Phosphate-buffered saline

487 PEN Polyethylene naphthalate

488 PFA Paraformaldehyde

489 PI3K-AKT Phosphoinositide 3-kinase-protein kinase B

490 qPCR Quantitative polymerase chain reaction

491 RANKL Receptor activator of nuclear factor-kappa B ligand

492 RFP Red fluorescent protein

493 RNA-seq RNA sequencing
494 RSEM RNA-Seq by Expectation-Maximization
495 RT-qPCR Reverse transcription quantitative polymerase chain reaction
496 SD Standard deviation
497 SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
498 SMART-seq2 Switching Mechanism at 5-prime End of RNA Template sequencing 2
499 SSC-A Side scatter area
500 T_CO Tumor cells isolated from osteoclast co-culture
501 T_T Osteoclastogenic cytokine-treated tumor cells
502 T_UT Untreated tumor cells
503 TAZ Transcriptional co-activator with PDZ-binding motif
504 TNF-alpha Tumor necrosis factor-alpha
505

506 Declarations

507 **Ethics approval and consent to participate**

508 All animal experiments were performed with approval from Department of Health under
509 Animals (Control of Experiments) Ordinance (Cap. 340) and approved by City University
510 of Hong Kong animal ethics committee. The analysis of publicly available, de-identified
511 human single-cell RNA-sequencing data did not involve the recruitment of new
512 participants or collection of new patient material.

513 **Consent for publication**

514 Not applicable

515 **Availability of data and materials**

516 The data supporting the findings of this study are available within the article and its
517 supplementary materials. The publicly available single-cell RNA-sequencing dataset
518 analyzed in this study is available from the Gene Expression Omnibus under accession
519 number GSE190772. Sequencing data generated in this study is available from the lead
520 author upon reasonable request.

521 **Competing interests**

522 The authors declare that they have no competing interests.

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531 **Authors' contributions**

532 K.H.L. and K.T.C. conceived and designed the study. K.H.L., D.S., and X.L. developed the
533 methodology. K.H.L., D.S., X.L., E.D., M.W., C.M., and Y.L. performed the experiments and
534 investigations. K.H.L., D.S., and X.L. prepared the visualizations. K.T.C. and X.W.
535 supervised the study. K.H.L. and K.T.C. wrote the manuscript. All authors read and
536 approved the final manuscript.

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FIGURE 1

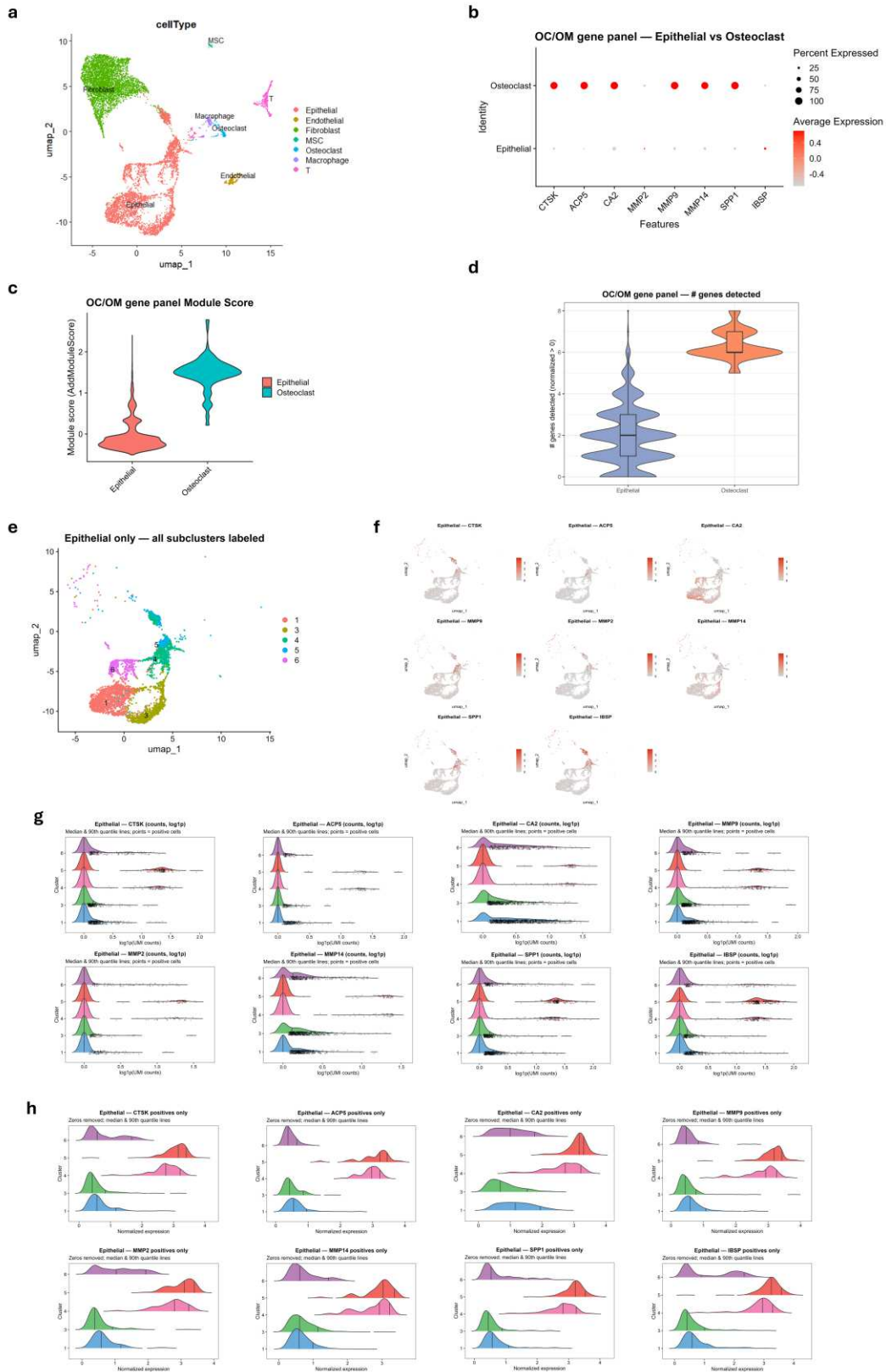


Fig. 1: Rare breast tumor subset display osteoclast-like gene signatures in breast cancer-bone metastases. **a.** UMAP for cell clusters defined by authors in GSE190772 containing single-cell RNA sequencing of bone metastases originated from left pelvis/right tibia, with cell types assigned based canonical marker identification and SingleR automatic cell type assignment. **b.** Dot plot comparing expression of an osteoclast/osteomimicry (OC/OM) gene panel between epithelial and osteoclast cells. Dot size represents the fraction of expressing cells, while color intensity indicates average expression. **c-d,** Comparison of OC/OM gene panel enrichment between epithelial and osteoclast cells, shown by module score (**c**) and number of detected OC/OM genes per cell (**d**). **e.** UMAP for epithelial cells only, showing epithelial subclusters defined from the author-provided clustering. **f.** Feature plots showing expression of individual OC/OM genes within epithelial cells. **g-h.** Ridge plots showing OC/OM gene expression distributions across epithelial subclusters using log-transformed counts (**g**) and positive cells only (**h**), highlighting epithelial subsets with detectable OC/OM-associated gene expression.

FIGURE 2

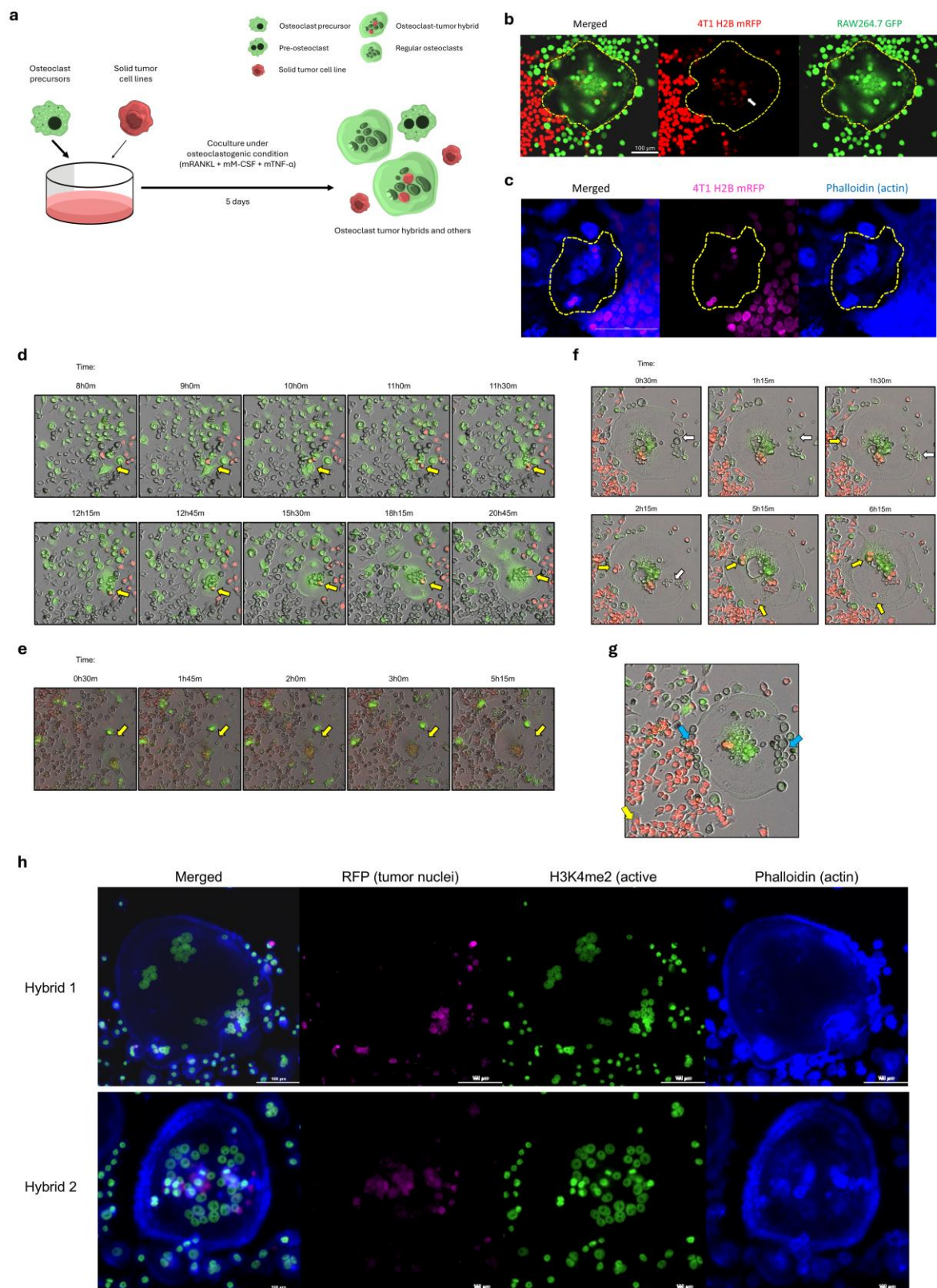


Fig. 2: Osteoclasts fuse with various solid tumors to form osteoclast-tumor hybrids *in vitro*.

a, Schematic diagram showing co-culture fusion assay between osteoclast precursors and solid tumor cell lines. **b**, Representative live cell images of a hybrid-like cell (yellow outline) formed between RAW264.7-GFP osteoclasts (green) and 4T1-H2B-mRFP breast cancer cells (red). Tumor nuclei expressed H2B-mRFP (white arrow) within osteoclast cytoplasm. Scale bar, 100 μ m. **c**, Representative live cell images of a hybrid-like cell (yellow outline) formed between a primary bone-marrow-derived osteoclast (actin outline) and 4T1-H2B-mRFP breast cancer cells (magenta). Scale bar, 100 μ m. **d-f**, Fluorescent time-lapse video showing cell fusion events between RAW264.7-GFP and 4T1-H2B-mRFP at various stages of osteoclast differentiation. **(d)** Tumor cell (yellow arrow) converged with other pre-osteoclasts to form a multinucleated osteoclast. Video stills were acquired from Movie S1. **(e)** Two osteoclast-tumor hybrids (yellow arrow) fused with each other. Video stills were acquired from Movie S2. **(f)** Individual tumor cells (yellow arrow) and pre-osteoclasts (white arrow) fused into an existing hybrid cell. Video stills were acquired from Movie S3. **g**, Live cell image showing RAW-246.7 GFP and 4T1-H2B-mRFP in distinct morphologies. Tumor and pre-osteoclast cells adjacent to an osteoclast displayed round cell shape (blue arrows) while tumor cells in growing colony were epithelial-shaped (yellow arrow). **h**, Representative immunofluorescent images for RAW264.7-GFP osteoclast-tumor hybrid-like cells stained for H3K4me2 transcriptional activity. Hybrids 1 and 2 contained tumor nuclei that fully colocalized with H3K4me2.

FIGURE 3

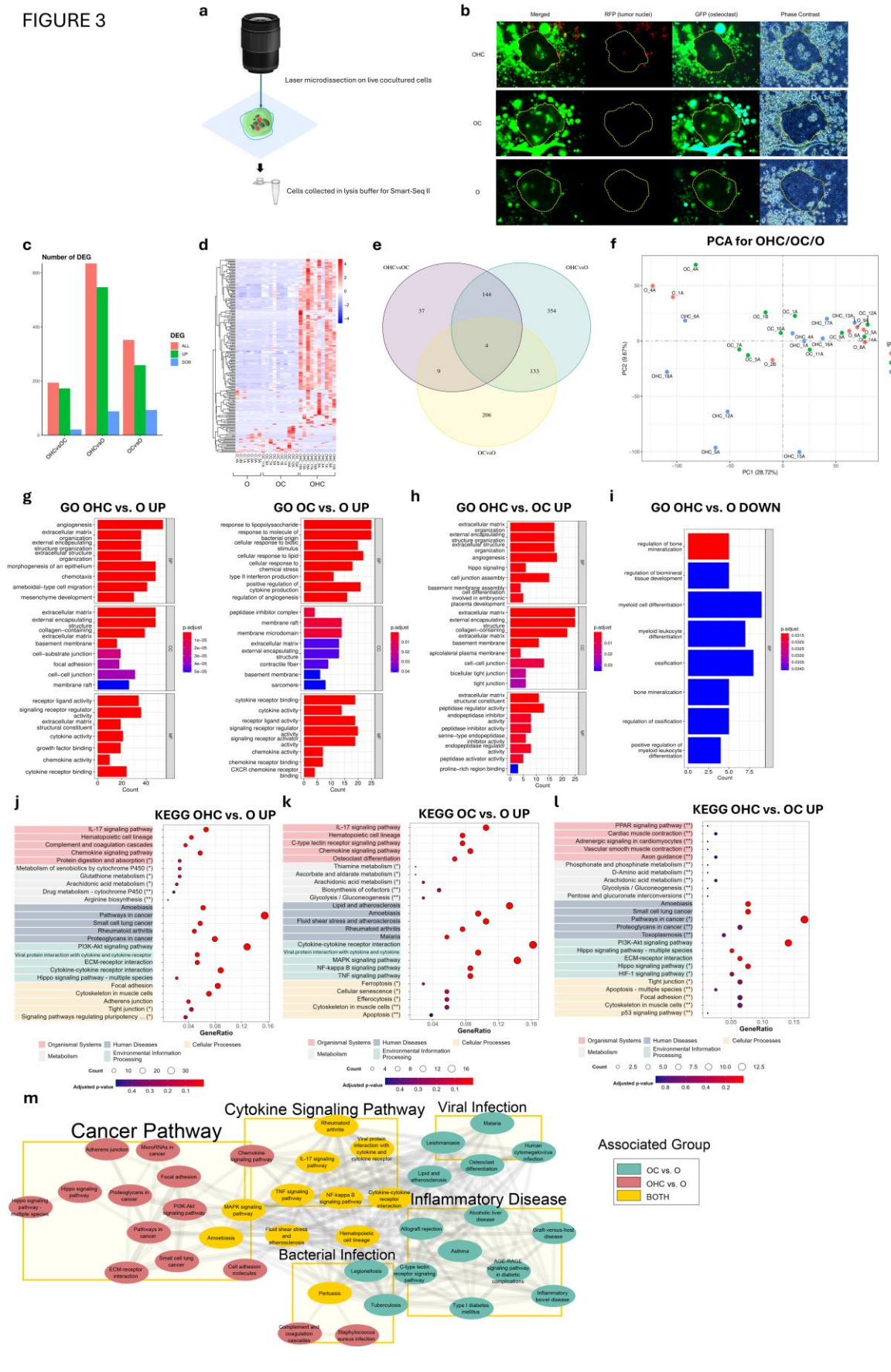


Fig. 3: Osteoclast-tumor hybrids are transcriptionally distinct from non-hybrid osteoclasts.

a, Schematic diagram displaying live cell laser microdissection protocol on RAW264.7-GFP osteoclasts co-cultured with 4T1-H2B-mRFP breast cancer cells to excise hybrids and other cells of interest for single cell sequencing. **b**, Representative fluorescent images for three osteoclast sample types with hybrids (OHC) (n = 10), non-hybrid osteoclasts from co-culture (OC) (n = 10), and non-hybrid osteoclasts from osteoclast-only culture (O) (n = 7) with osteoclasts highlighted. **c**, Bar graph showing three DEGs comparisons with OHC vs. O, OC vs. O, and OHC vs. OC. Upregulated, downregulated, and overall DEGs were displayed for each group. **d**, Heatmap displaying both upregulated and downregulated DEGs amongst the three osteoclast sample types. **e**, Venn diagram showing overlapping and unique overall DEGs amongst the three osteoclast comparisons. **f**, PCA analysis for individual samples from three osteoclast sample types. **g,h** Gene ontology (GO) analyses for hybrids and non-hybrid osteoclasts on three comparisons (OHC vs. O, OC vs. O, and OHC vs. OC). Upregulated gene functions on biological processes (BP), cellular components (CC), and molecular functions (MF) for **(g)** OHC vs. O, OC vs. O, and **(h)** OHC vs. OC. **(i)** Attenuated gene functions on BP, CC, and MF for OHC vs. O. p.adjusted value < 0.05. **j-l**, KEGG pathway analyses for the same comparison groups. Top five pathways augmented in various categories for **(j)** OHC vs. O, **(k)** OC vs. O, and **(l)** OHC vs. OC. Pathways were shown in order of significance with following annotations: no asterisk – p.adjusted value & p value < 0.05, one asterisk (*) – p.adjusted value > 0.05 & p value < 0.05, two asterisks (**) – p. adjusted value & p value > 0.05. **(m)** Network analysis for significant pathways enriched in OHC vs. O (red), OC vs. O (blue), and both (yellow). p.adjusted value < 0.05.

FIGURE 4

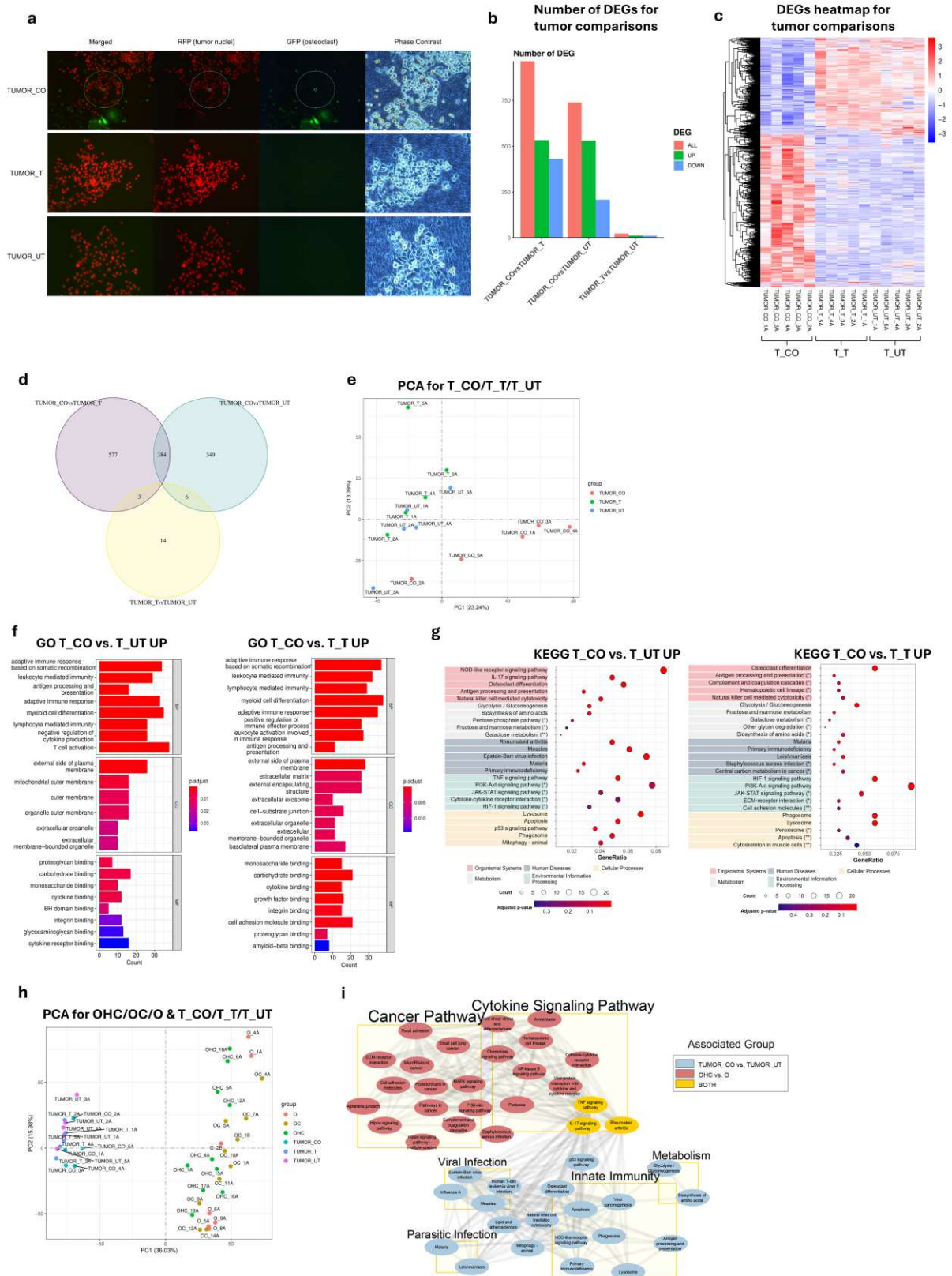


Fig. 4: Breast cancer cells express unique gene expression profile in the presence of osteoclasts. **a**, Representative fluorescent images for three tumor sample types with tumors from co-culture (TUMOR_CO) (T_CO), osteoclastogenic cytokine-treated tumors (TUMOR_T) (T_T), untreated tumors (TUMOR_UT) (T_UT). Example for T_CO isolated cells were highlighted in circle. **b**, Bar graph showing three DEGs comparison groups with TUMOR_CO vs. TUMOR_T, TUMOR_CO vs. TUMOR_UT, and TUMOR_T vs. TUMOR_UT. **c**, Heatmap containing both upregulated and downregulated DEGs amongst the three tumor sample types. **d**, Venn diagram depicting overlapping and unique overall DEGs amongst the three tumor comparison groups. **e**, PCA analysis plots for individual tumors from three tumor sample types. **f**, GO analyses for tumor samples with three comparison groups (T_CO vs. T_UT and T_CO vs. T_T). Upregulated gene functions on BP, CC, and MF for T_CO vs. T_UT, and T_CO vs. T_T. **g**, KEGG pathway analyses for the same tumor comparison groups. Top five pathways augmented in various categories for T_CO vs. T_UT and T_CO vs. T_T. Pathways were shown in order of significance with following annotations: no asterisk – p.adjusted value & p value < 0.05, one asterisk (*) – p.adjusted value > 0.05 & p value < 0.05, two asterisks (**) – p. adjusted value & p value > 0.05. **h**, PCA analysis plots for individual samples from both osteoclast and tumor sample types. **i**, Network analysis showing significant pathways enriched in T_CO vs. T_UT (light blue), OHC vs. O (red), and both (yellow). p.adjusted value < 0.05.

FIGURE 5

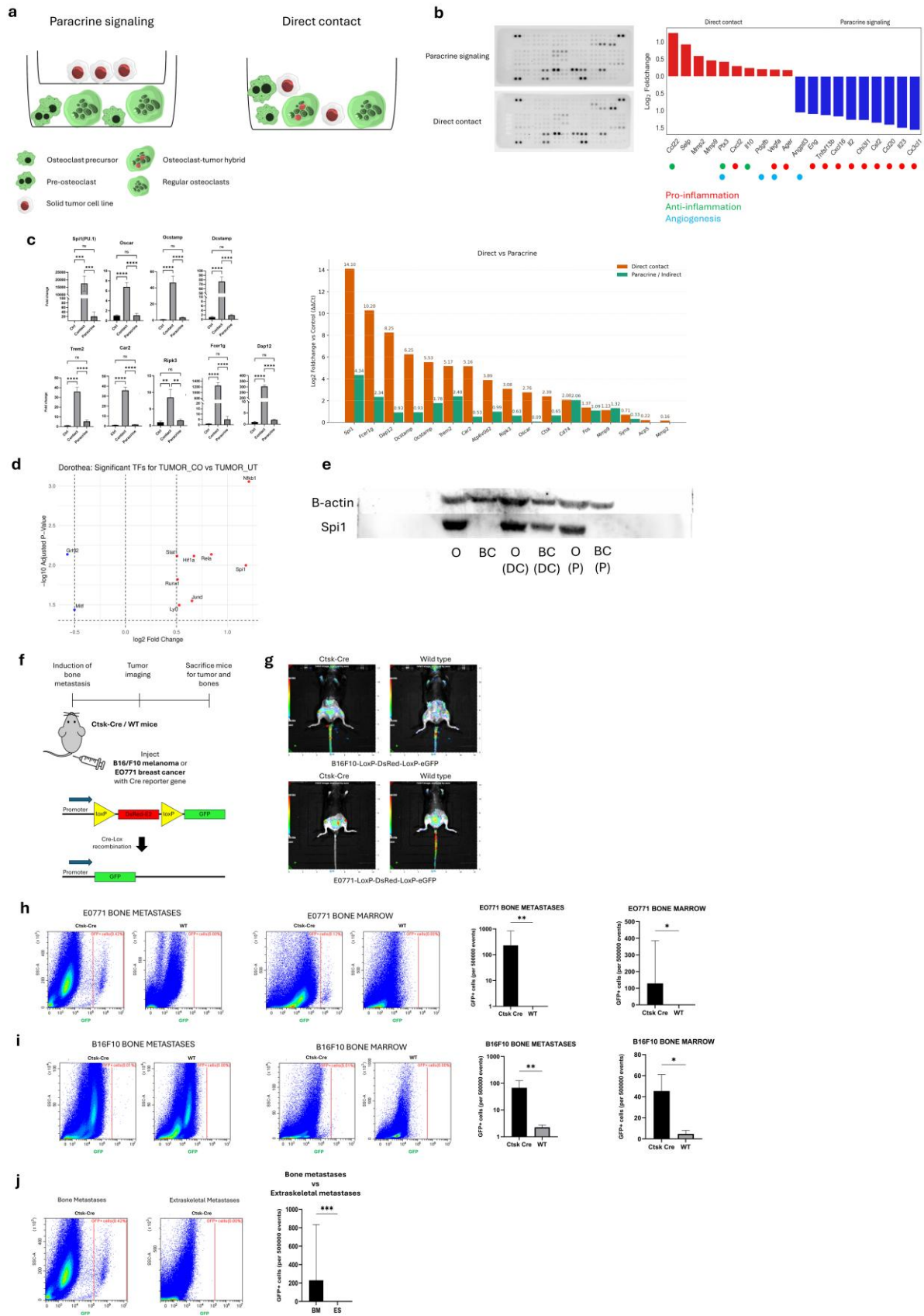


Fig. 5: Direct contact with osteoclasts reprograms breast cancer cell transcriptome. **a**, Schematic of indirect (paracrine) and direct co-cultures of RAW264.7 pre-osteoclasts and 4T1 breast cancer cells. **b**, Cytokine-array blots and the ten most differentially detected cytokines between direct and paracrine co-cultures, categorized as pro-inflammatory (red), anti-inflammatory (green), or angiogenic (blue). No statistical testing was performed. **c**, qPCR analysis of 4T1 cells isolated from direct and paracrine co-cultures, with conventionally cultured 4T1 cells as the baseline control. **d**, Dorothea analysis of TUMOR_CO versus TUMOR_UT showing differentially activated transcription factors. **e**, Western blot analysis of RAW264.7-derived osteoclasts and 4T1 cells cultured alone or isolated from direct and paracrine co-cultures: osteoclasts alone, O; breast cancer cells alone, BC; osteoclasts from direct co-culture, O (DC); breast cancer cells from direct co-culture, BC (DC); osteoclasts from paracrine co-culture, O (P); and breast cancer cells from paracrine co-culture, BC (P). **f**, Schematic of bone metastasis induction in Ctsk-Cre and wild-type (WT) mice using EO771 breast cancer or B16F10 melanoma cells expressing a DsRed-E2-to-GFP Cre reporter. **g**, DsRed-E2 fluorescence in mice with EO771 or B16F10 bone metastases. **h**, **i**, Flow cytometric detection and quantification of DsRed-E2-positive and Cre-recombined GFP-positive tumor cells in bone metastases and bone marrow. EO771: bone metastases, Ctsk-Cre (n = 8) and WT (n = 5); bone marrow, Ctsk-Cre (n = 5) and WT (n = 4). B16F10: bone metastases, Ctsk-Cre (n = 6) and WT (n = 4); bone marrow, Ctsk-Cre (n = 5) and WT (n = 3). **j**, Quantification of GFP-positive recombined cells in EO771 bone metastases (n = 8) and extraskeletal metastases (n = 6). GFP-positive cells were quantified per 500,000 events. Data are presented as mean $\pm$ s.d. $P < 0.05$, $P < 0.01$, and $P < 0.001$.

FIGURE 6

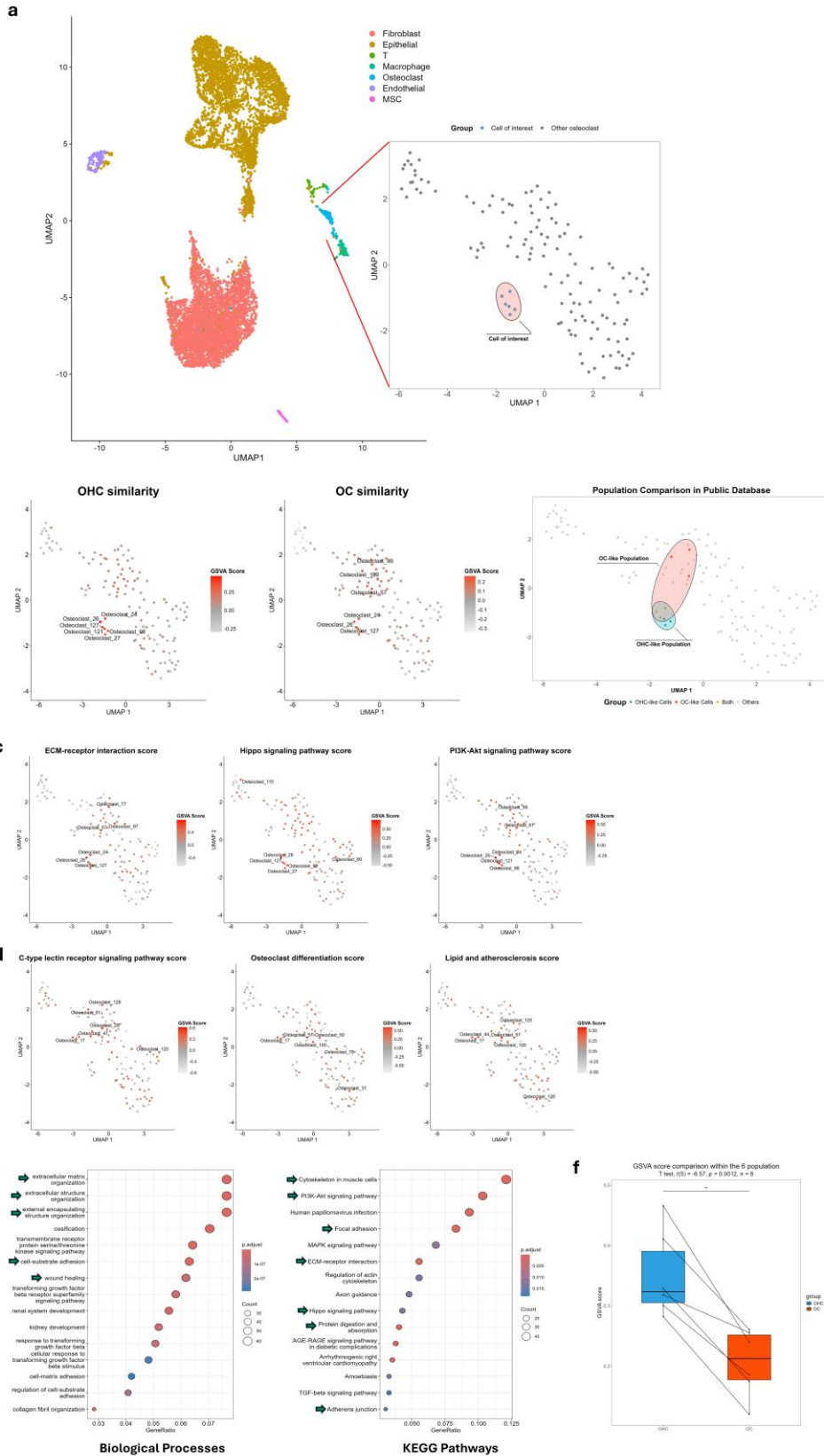
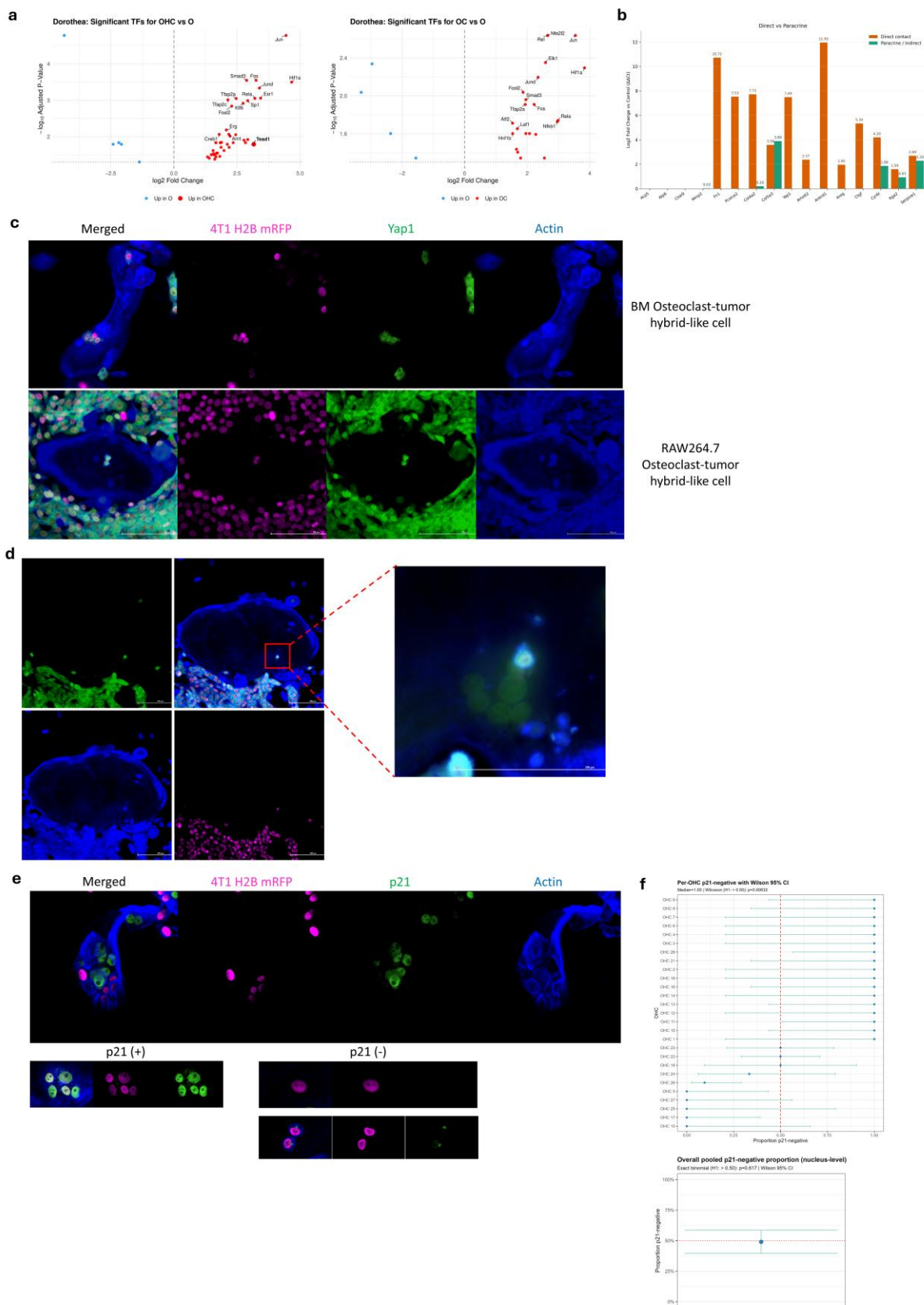


Fig. 6: Human ER+ breast cancer-bone metastasis contains cells resembling osteoclast-tumor hybrids and tumors from co-culture. **a**, Cells of interest resembling hybrid-like cells in osteoclast population pre-defined by authors from GSE190772 containing scRNA data derived from ER+ human patient breast cancer-bone metastasis. **b**, Gene set variation analyses (GSVA) for overall DEGs derived from OHC vs. O and OC vs. O mapped and compared against osteoclast population. Similarity scores for OHC vs. O and OC vs. O plotted across osteoclast population with top six scores labelled on individual osteoclasts. Individual osteoclast cells in GSVA with scores > 0 were highlighted in ascending red intensity. Expected OHC- (red) and OC-like (blue) populations were interpolated as highlighted clusters within osteoclast population. **c,d**, GSVA for several unique upregulated pathways in **(c)** OHC vs. O and **(d)** OC vs. O respectively. **e**, GO (BP) and KEGG pathway breakdown on OHC-like cluster with several highlighted functions and pathways resembling hybrid-like cells in our data (green arrows). **f**, GSVA score comparison for OHC vs. O and OC vs. O centroids for OHC-like cell cluster. Statistical analysis via paired T test (n = 6); p = 0.0012.

FIGURE 7



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Fig. 7: Yap1-activated osteoclasts exhibit a proliferation-permissive state. **a**, Dorothea analyses for OHC vs. O and OC vs. O highlighted Yap1-dependent TEAD1 transcription factor expression in hybrid-like cells. **b**, RAW264.7 osteoclasts from direct co-culture with 4T1 breast cancer cells promoted Yap1 downstream gene activation (Amotl2, Ankrd1, Ctgf, Cyr6l, Itga2, Serpine1) and enhanced ECM-modifying factors (Fn1, Pcolce2, Col4a2, Col5a3). **c**, Tumor nuclei present in primary and RAW264.7 hybrid-like cells displayed nuclear colocalized Yap1. **d**, Hybrid-like cell containing tumor nucleus with colocalized Yap1 were located adjacent to osteoclast nuclei expressing low Yap1 signals. **e**, Hybrid-like cells stained for p21 cell cycle inhibitor with p21-colocalized nuclei indicated as p21-positive (+) and non-p21-colocalized nuclei indicated as p21-negative (-). **f**, OHC-level quantification of p21-negative tumor nuclei, plotted as the fraction of p21-negative tumor nuclei per individual OHC with Wilson 95% confidence intervals; significance versus 50% was assessed by one-sample Wilcoxon signed-rank test ($p = 0.00632$). **g**, Nucleus-level quantification of p21-negative tumor nuclei pooled across OHCs, plotted as the overall p21-negative fraction with Wilson 95% confidence intervals; significance versus 50% was assessed by exact binomial test ($p = 0.617$).

FIGURE S1

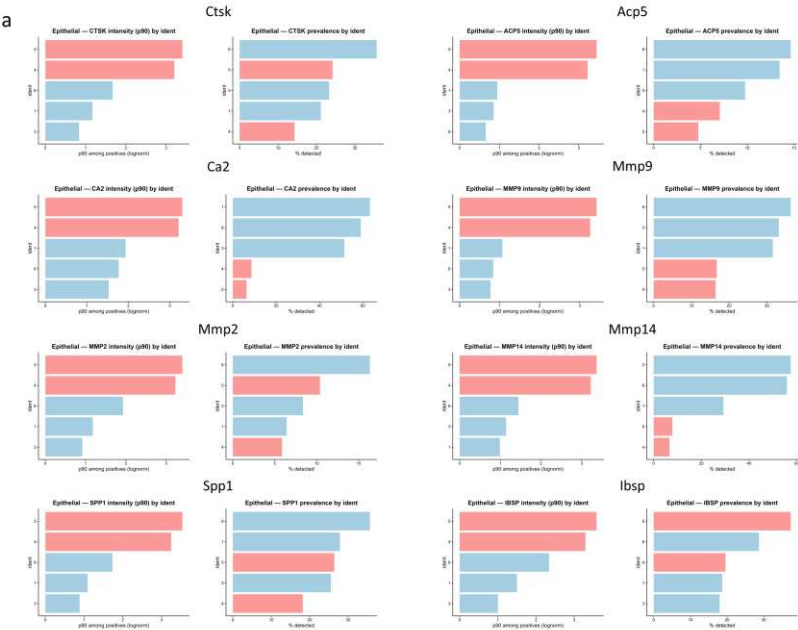


Fig. S1. OC/OM gene-set prevalence and intensity across epithelial subclusters.
a, Prevalence (% detected) and intensity (p90 among positive cells) of OC/OM-associated genes in epithelial subclusters 1, 3, 4, 5, and 6. Genes shown include CTSK, ACP5, CA2, MMP9, MMP2, MMP14, SPP1, and IBSP. Statistical significance was assessed using the Wilcoxon rank-sum test.

FIGURE S2

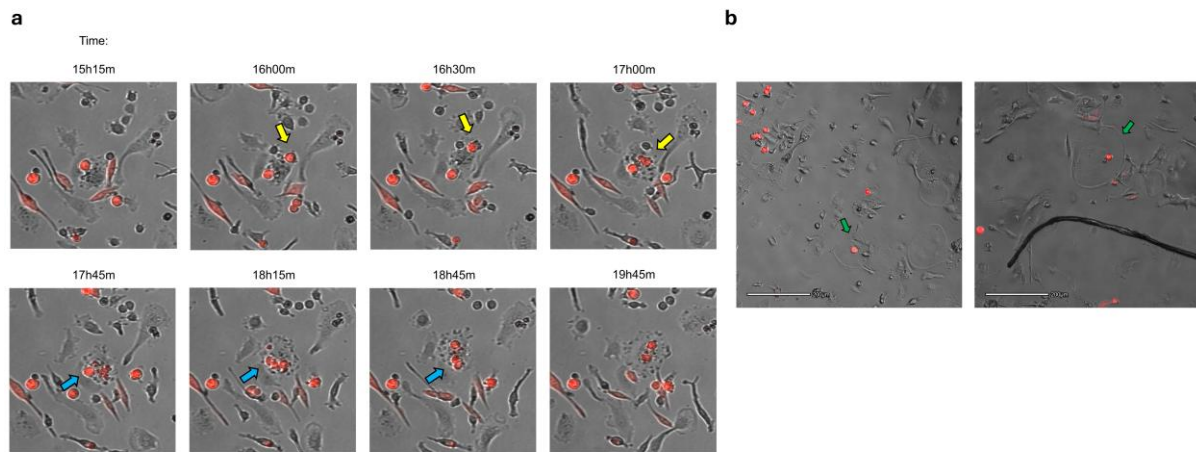


Fig. S2. Primary osteoclasts fuse with breast cancer cells *in vitro*. a, Primary osteoclast acquiring EO771 Cre reporter and their nuclei into its cytoplasm (yellow & blue arrows). Video stills were acquired from Movie S4. b, Examples of EO771 Cre reporter tumor nuclei present inside developing primary osteoclasts (green arrows).

FIGURE S3

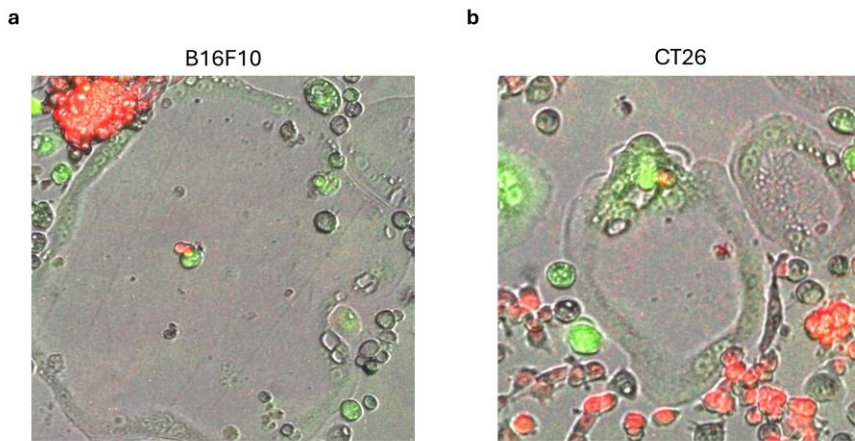


Fig. S3. Osteoclasts fuse with melanoma and colon cancer cells *in vitro*. a,b, Fluorescent snapshot image for osteoclast-tumor hybrid-like cells formed between RAW264.7-GFP and other solid tumor cell lines. Hybrids containing tumor nuclei derived from (a) B16F10-H2B-mRFP melanoma and hybrids with tumor nuclei derived from (b) CT26-H2B-mRFP colon cancer.

FIGURE S4

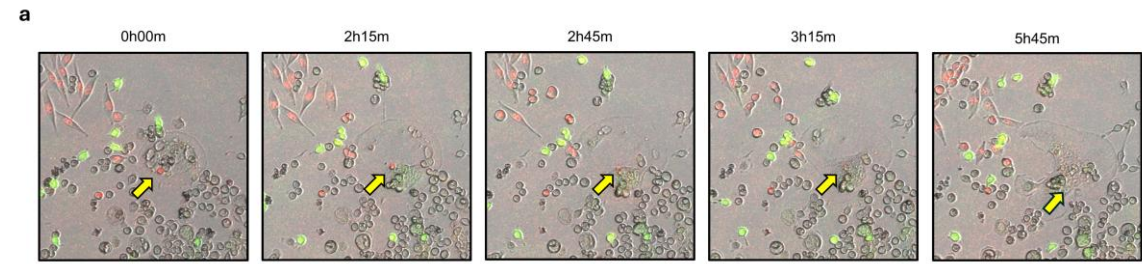


Fig. S4. Cell-in-cell phenomenon between tumor and osteoclast. a, Tumor nucleus migrated near osteoclast nuclei and showed signs resembling disintegration by entosis with declining tumor fluorescent signal (yellow arrow). Video stills were acquired from Movie S5.

FIGURE S5

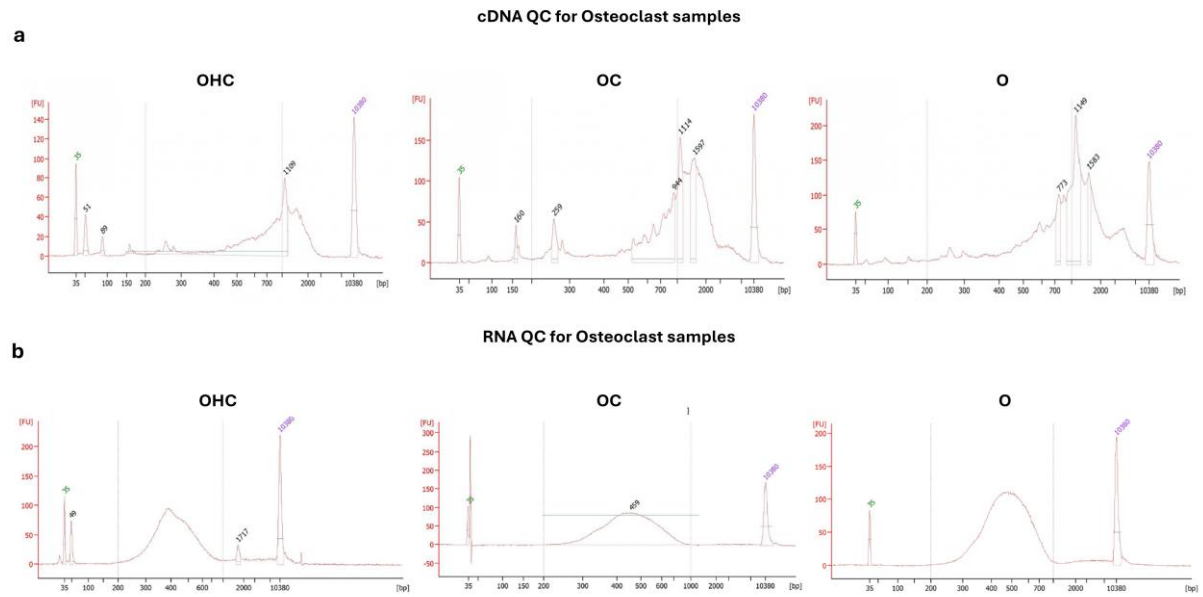


Fig. S5. RNA quality control for osteoclast variants. a,b, cDNA and library QC graphs for three osteoclast sample types. (a) Representative cDNA QC graph for OHC, OC, and O. (b) Representative library QC graph for OHC, OC, and O.

FIGURE S6

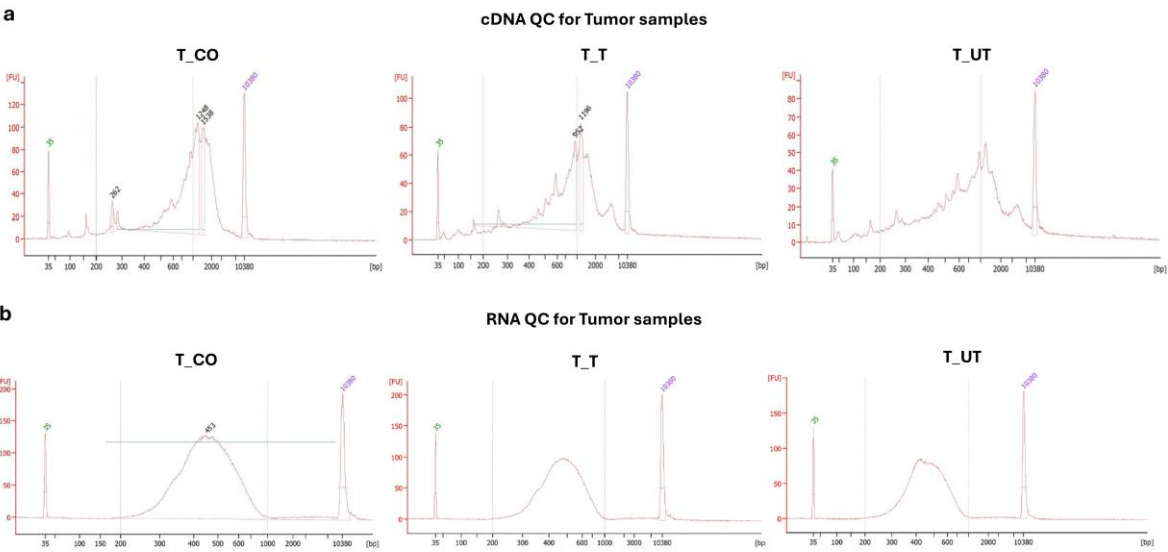


Fig. S6. RNA quality control for tumor variants. a,b, cDNA and library QC graphs for three tumor sample types. (a) Representative cDNA QC graph for TUMOR_CO, TUMOR_T, and TUMOR_UT. (b) Representative library QC graph for TUMOR_CO, TUMOR_T, and TUMOR_UT.

FIGURE S7

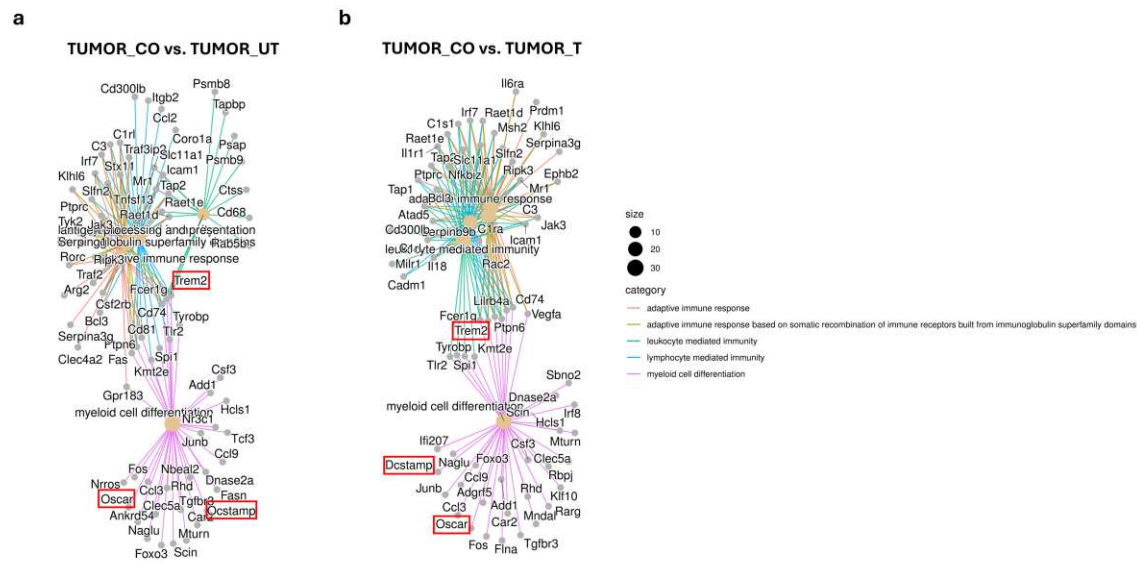


Fig. S7. Gene ontology network analysis for DEGs in TUMOR_CO vs. TUMOR_UT and TUMOR_CO vs. TUMOR_T. a,b, GO network for DEGs from (a) TUMOR_CO vs. TUMOR_UT and (b) TUMOR_CO vs. TUMOR_T under different categories. Notable genes relevant to osteoclast differentiation and fusion were highlighted in red boxes.

FIGURE S8

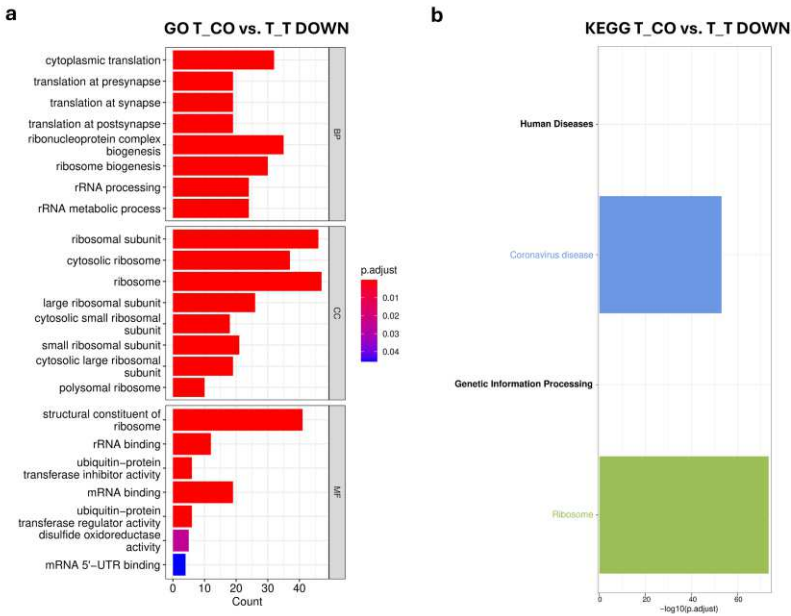


Fig. S8. Attenuated genes and pathways in TUMOR_CO vs. TUMOR_T. a, GO analysis for downregulated DEGs in TUMOR_CO vs. TUMOR_T. GO categories shown included BP, CC, and MF. p.adjusted value < 0.05. b, KEGG pathway analysis for attenuated pathways in TUMOR_CO vs. TUMOR_T. p.adjusted value < 0.05.

FIGURE S9

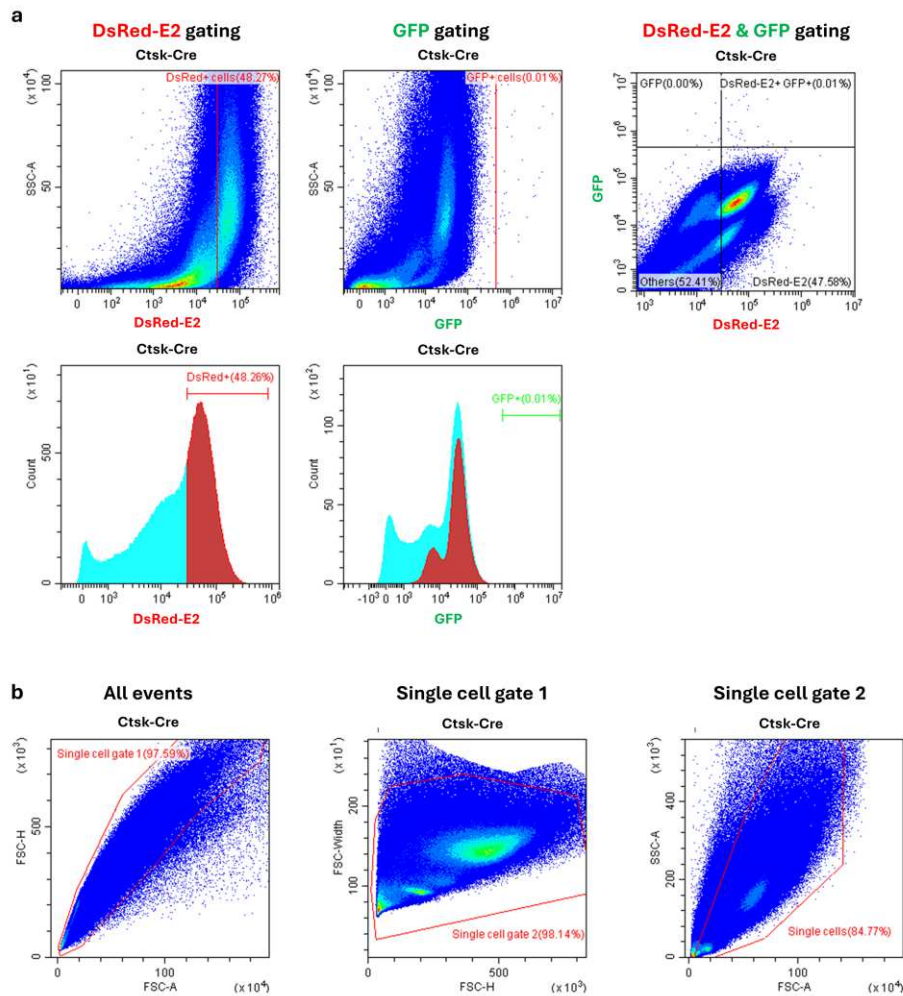


Fig. S9. Flow cytometry analysis on bone metastases in Ctsk-Cre mice and single cell gating strategies. a, Flow cytometry analysis on gated single cells showing DsRed-E2+ and GFP+ cells in bone metastases derived from Ctsk-Cre mice. b, Single cell gating strategy on cells isolated from bone metastases. Doublet cells were gated out twice through FSC-A vs. FSC-H and FSC-H vs. FSC-W gates in Single cell gate 1 & 2 respectively. Cellular debris with low FSC-A & high SSC-A was gated out in Single cell gate 2 to acquire single cells for analysis.

1273 **Supp. Table 1. Primer sequences used for quantitative RT–PCR**

Gene symbol	Forward primer (5′–3′)	Reverse primer (5′–3′)
Spi1	TTACAGGCGTGCAAAATGGAA	GACGTTGGTATAGCTCTGAATCG
Fcer1g	ATCTCAGCCGTGATCTTGTTCT	ACCATACAAAAACAGGACAGCAT
Dap12 (Tyrobp)	CCCAAGATGCGACTGTTCTTC	GTCCCTTGACCTCGGGAGA
Dcstamp	GGGGACTTATGTGTTTCCACG	ACAAAGCAACAGACTCCCAAAT
Ocstamp	CTGTAACGAACACTGACCCAGC	CCAGGCTTAGGAAGACGAAGA
Trem2	CTGGAACCGTCACCATCACTC	CGAAACTCGATGACTCCTCGG
Car2	GATAAAGCTGCGTCCAAGAGC	GCATTGTCCTGAGAGTCATCAA
Atp6v0d2	CTGGTTCGAGGATGCAAAGC	GTTGCCATAGTCCGTGGTCTG
Ripk3	GTGCTACCTACACAGCTTGAAC	CCCTCCCTGAAACGTGGAC
Oscar	CCTAGCCTCATACCCCAG	CGTTGATCCCAGGAGTCACAA
Ctsk	CTCGGCGTTTAATTTGGGAGA	TCGAGAGGGAGGTATTCTGAGT
Cd74	AGATGCGGATGGCTACTCC	TCATGTTGCCGTACTTGGTAAC
Fos	CGGGTTTCAACGCCGACTA	TGGCACTAGAGACGGACAGAT
Mmp9	GCAGAGGCATACTTGTACCG	TGATGTTATGATGGTCCCACTTG
Syna	ATGGTTCGTCCTTGGGTTTTTC	GTGTTGAGTGAGGTTTACCAGG
Acp5	CACTCCCACCCTGAGATTTGT	CCCCAGAGACATGATGAAGTCA
Mmp2	ACCTGAACACTTTCTATGGCTG	CTTCCGCATGGTCTCGATG

1274
1275
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1278 Movies

1279 **Movie S1. Live imaging of cell fusion between several pre-osteoclasts and an individual**
1280 **tumor cell.** 4T1-H2B-mRFP breast cancer cell fused and converged with RAW264.7-GFP pre-
1281 osteoclasts to form an osteoclast-tumor hybrid-like cell with intact tumor nucleus. Video was
1282 captured using CytoSMART Lux3 FL fluorescent time-lapse recording function. Video stills for
1283 Movie S1 were used in Fig. 2d.

1284

1285 **Movie S2. Live imaging of cell fusion between two osteoclast-tumor hybrids.** Two
1286 osteoclast-tumor hybrid-like cells fused with each other upon making cell membrane contact
1287 to form an enlarged hybrid-like cell. Video was captured using CytoSMART Lux3 FL fluorescent
1288 time-lapse recording function. Video stills for Movie S2 were used in Fig. 2e.

1289

1290 **Movie S3. Live imaging of individual tumor cells fusing into an existing osteoclast-tumor**
1291 **hybrid.** 4T1-H2B-mRFP breast cancer cells and RAW264.7-GFP fused and incorporated their
1292 nuclei into an existing osteoclast-tumor hybrid-like cell. Video was captured using CytoSMART
1293 Lux3 FL fluorescent time-lapse recording function. Video stills for Movie S3 were used in Fig.
1294 2f.

1295

1296 **Movie S4. Live imaging of cell fusion between primary osteoclasts and breast cancer cells.**
1297 EO771 Cre reporter (red fluorescence) fused with primary BM-derived osteoclasts
1298 differentiated using BM cells from Ctsk-Cre mice. Video was captured using CytoSMART Lux3
1299 FL fluorescent time-lapse recording function. Video stills for Movie S4 were used in Fig. S2a,
1300 b.

1301

1302 **Movie S5. Live imaging of tumor nucleus activity in a hybrid-like cell post-fusion.** EO771-
1303 H2B-mRFP tumor nucleus disintegrated adjacent to osteoclast nuclei in an osteoclast-tumor
1304 hybrid-like cell. The osteoclast proceeded to split into 3 daughter osteomorphs. Video was
1305 captured using CytoSMART Lux3 FL fluorescent time-lapse recording function. Video stills for
1306 Movie S5 were used in Fig. S4a.

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1308

1309

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [MovieS1.mp4](#)
- [MovieS2.mp4](#)
- [MovieS3.mp4](#)
- [MovieS4.mp4](#)
- [MovieS5.mp4](#)
- [Westernblotuncroppedlabeled.tif](#)