

The Role of Alveolar Pneumocytes in the Lung Microenvironment of Osteosarcoma Metastases

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Abstract

Pulmonary metastasis is the leading cause of mortality in human and canine osteosarcoma (OS) patients. However, the contribution of lung-resident epithelial cells, particularly surfactant protein C (SPC) expressing type II alveolar pneumocytes (TIIPs), to metastatic progression remains under-defined. This study investigates the spatial distribution of pneumocytes in OS lung metastases and explores their interactions with tumor cells. We show that TIIPs accumulate in the tumor periphery of OS pulmonary metastases in human and canine patients as well as in an OS murine model of spontaneous metastases. In an *in vitro* setting, TIIP co-culture or TIIP conditioned media significantly increased OS cell proliferation. RNAseq analysis of TIIPs in co-culture with OS cells showed a significant enrichment of the p53 pathway, underpinning injury/stress response in TIIPs. The secretome in the co-culture system consisted of pro-fibrotic mediators, including GDF-15, Dkk-1, VEGF, and TGF- β 1. This investigation highlights a previously under-recognized epithelial component of the OS metastatic microenvironment and suggests that TIIPs contribute to a growth-permissive, pro-fibrotic metastatic niche. Future work aims to identify new approaches for targeting host-tumor interactions to limit metastatic progression of OS in both human and canine patients.

Introduction

Osteosarcoma (OS) is the most prevalent primary malignant bone tumor in children and adolescents as well as companion large breed dogs [1–3]. The survival rate for patients with localized disease is approximately 70%, with standard of care comprising multimodal chemotherapy alongside surgery [4]. Metastasis is the leading cause of mortality in human and canine osteosarcoma patients; the 10-year survival rate remains below 25% in humans and below 10% in dogs at three years post-diagnosis [5]. Pulmonary metastasis is common in both species, accounting for 90% of metastatic sites, making it the leading cause of osteosarcoma-associated death in patients with incurable disease [5]. It is estimated that over 80% of OS patients harbor undetectable lung micro-metastases at the time of initial diagnosis. Once metastatic disease is clinically evident, current therapies offer marginal benefit [6]. The lack of predictive biomarkers for therapy selection and patient stratification further compounds this challenge [7, 8]. The striking similarities between canine and human OS in clinical presentation, biologic behavior, natural disease course, molecular landscape, and genomic architecture collectively underscore the comparative relevance of the canine patient model for both discovery research and therapeutic investigation [9–11]. Previously, we explored the role of stromal cells and immune cells in osteosarcoma pulmonary metastasis across human and canine patients and observed that the role of lung resident epithelial cells remains relatively under-defined [12].

Mechanistically, the development of pulmonary metastasis is a complex and multi-step cascade that is fundamentally distinct from primary tumor formation; disseminated OS cells must traverse the basement membrane, survive transit in the circulation, evade immunosurveillance, seed within the alveolar space, and ultimately remodel the local microenvironment into a niche permissive for outgrowth. Each step is governed by reciprocal interactions between cancer cells and the diverse array

of cell types in the tumor microenvironment (TME) embedded within a dynamically remodeling extracellular matrix (ECM) [13, 14]. The *de novo* cellular and extracellular environment supports cancer cell heterogeneity, clonal expansion, immune evasion, and the emergence of drug resistance. Increasing evidence positions the TME not merely as a passive backdrop to tumor growth, but as an active and critical determinant of metastatic outcomes and therapeutic failure [15–17].

Upon dissemination, OS cells often seed in the alveolar space of the lung, a microenvironment dominated by two functionally distinct epithelial cell populations: type I pneumocytes (TIPs) and type II pneumocytes (TIIPs). TIPs are flattened cells, covering approximately 96% of the alveolar surface and are principally responsible for gas exchange. TIIPs are large, cuboidal cells with microvilli on their apical surface, comprising 4% of the alveolar surface area, yet they perform a disproportionate range of functions that have earned them the designation "defenders of the alveolus" [18]. Their three primary roles are: (1) maintenance of alveolar surface tension through the synthesis, storage, and secretion of pulmonary surfactant protein C (SPC); (2) serving as the resident stem cells of the alveolar epithelium, capable of differentiating into TIPs following injury or stress; and (3) functioning as master regulators of innate host defense through direct and indirect interactions with immune effectors including macrophages, neutrophils, eosinophils, and multiple T cell subsets [19, 20]. Therefore, TIIPs play important roles in normal pulmonary function and damage response following injury/stress.

Recent studies have begun to shed light on the involvement of alveolar epithelial cells in pulmonary metastatic progression of other cancers. For example, in breast cancer pulmonary metastases, TIPs maintained metastatic cells in an indolent state through an integrin-dependent pro-survival mechanism and activated TIIP cells induced reactivation of dormant tumor cells via TGF- β 1/SNAI2 signaling [21, 22]. However, the precise interactions of pneumocytes with osteosarcoma pulmonary metastases remain poorly understood. This study aims to characterize the spatial distribution of pneumocytes in the TME of OS lung metastases and to elucidate the molecular and secretory profiles governing the resident lung cell-cancer cell crosstalk, including the resultant effects on osteosarcoma cell proliferation. Together, these aims provide a multi-dimensional view of resident lung epithelial cell involvement in OS pulmonary metastasis to identify candidate mechanisms as therapeutic targets for future investigation.

Methods

Spatial profiling

To determine the distribution of pneumocytes in the TME of human OS lung metastases, a spatial transcriptomics dataset was queried [23] ($n = 7$, **Supplementary Table 1**). Spatial feature plots were derived using Seurat for the relative enrichment of aggregate TIP and TIIP metagene signatures derived from the LungMap project (<https://www.lungmap.net>). The TIP gene signature comprised: *HOPX*, *AGER*, *RTKN2*, *EMP2*, *CLDN18*, *LMO7*, *CLIC3*, *KRT7* and the TIIP gene signature comprised: *SFTPC*, *LAMP3*, *SLC34A2*, *SFTPB*, *SFTPA1*, *NPC2*, *NAPSA*. Additionally, spatial feature plots for TIIP marker *SFTPC* (SPC gene) were generated (**Supplementary Fig. 1**).

Immunohistochemistry (IHC)

Canine lung samples with metastatic OS (n = 14, **Supplementary Table 2**) were obtained from a large multisite clinical trial conducted through the NCI's Comparative Oncology Trials Consortium [24]. All trial activities were conducted under Institutional Animal Care and Use Committee approval at each participating veterinary institution. Murine lung tissue was collected at day 28 from NSG mice that developed spontaneous lung metastases following paratibial injection of 2×10^6 K7M2-pEGFP murine OS cells (n = 9; Frederick, NIH) [3]. All experimental procedures involving mice were performed in compliance with the NIH Animal Care and Use Committee guidelines under an approved protocol (PB-038). The lung tissue FFPE specimens for both species were probed for SPC expression by IHC to assess the abundance and location of TIIPs in TME. Canine samples were processed by VitroVivo Biotech. Sections were deparaffinized with xylene and rehydrated through graded ethanol. Antigens were retrieved with citrate buffer at 95°C for 15 minutes and quenched with 1% hydrogen peroxide in PBS for 15 minutes before blocking in Normal Goat Serum. Prosurfactant protein C primary antibody (#ab90716, abcam; 1:2000) was incubated overnight at 4°C. The next day, VisUCyte HRP goat anti-rabbit polymer reagent (#VC003, R&D Systems) as the secondary antibody was applied for 60 minutes at RT. Antigen-antibody complexes were visualized using DAB chromogen solution for 2 minutes. Sections were counterstained with Mayer's hematoxylin for 1 minute, dehydrated, and coverslipped. Positive and negative controls were incorporated in this protocol (**Supplementary Fig. 2**). For the murine samples, IHC staining was performed on LeicaBiosystems' BondRX autostainer with the following conditions: Epitope Retrieval 1 (Citrate) 20 minutes, Prosurfactant protein C (#ab90716, Abcam; 1:1000, 30 minutes), and the Bond Polymer Refine Detection Kit (#DS9800, LeicaBiosystems) minus the PostPrimary reagent. Positive and isotype controls were incorporated in this protocol (**Supplementary Fig. 3**). Slides were removed from the Bond autostainer, dehydrated through ethanols, cleared with xylenes, and coverslipped. Slides were digitally scanned into HALO (Indica Labs) for quantitative image analysis of SPC+ cells using the CytoNuclear algorithm v2.0.9. For this study, SPC+ staining was quantified in the non-tumor:tumor interface (NT:T) as well as the non-tumor/adjacent normal lung tissue (NT). To define NT:T regions, tumor regions were annotated, and a circle was drawn 200 μ m from the edge of the tumor annotation. NT regions were defined as regions greater than 200 μ m from the tumor annotation.

Cell culture

Human alveolar type II epithelial cells (TIIP, #ABC-TC5515) were purchased from AcceGen Biotechnology. The TIIPs were cultured in the TIIP medium kit as per the manufacturer's recommendation (#ABM-TM5515, AcceGen Biotechnology). Correlation analysis in R 4.5.2 using "corrplot" package confirmed a high correlation between RNAseq for TIIPs utilized for this study (n = 3) and publicly available single-cell RNAseq data of TIIPs isolated from two donors and grown in culture (LungMap project, (<https://www.lungmap.net>); $r = 0.79$, **Supplemental Fig. 4**). MG63.2 OS cells [25] were cultured in DMEM (#SH30285.01, Cytiva) supplemented with 10% FBS (#SH30071.03, Cytiva), and 1%

Penicillin-Streptomycin-Glutamine (#10378016, Gibco). All cells were maintained at 37°C in a humidified incubator with 5% CO₂.

Cell proliferation assay

To determine OS cell proliferation, an MTS-based assay was performed using the CellTiter 96 aqueous one solution cell proliferation assay. For the first experiment involving indirect co-culture, 2×10^4 TIIPs/well and 1×10^4 MG63.2 cells/well were plated in a 96-well Transwell® plate (#3381, Corning; TIIP – top and OS – bottom). The cells were cultured separately in their respective growth media for 24 H at 37°C and 5% CO₂. Media were then replaced with a 1:1 mixture of TIIP media and MG63.2 media to initiate co-culture. For the second experiment involving conditioned media, 1×10^4 MG63.2 cells/well were seeded in their growth media in a 96-well plate (#167542, ThermoFisher Scientific) and incubated at 37°C and 5% CO₂. After 24 H, media was replaced with either fresh MG63.2 growth media, TIIP unconditioned media, or TIIP conditioned media. For both experiments, 72 H post incubation, cell proliferation was determined by recording absorbance on the SpectraMax iD3 (Molecular Devices) at 490 nm after the addition of CellTiter 96 Aqueous One Solution (1:5 dilution, G3581, Promega) and incubation for 1 H at 37°C and 5% CO₂. Data were normalized to MG63.2 cells growing in OS media. Confluence was additionally monitored using the IncuCyte S3 live cell imaging system (Sartorius).

Co-culture of primary TIIPs and OS cells (TIIP-OS)

To further assess TIIP-OS crosstalk, primary TIIPs were co-cultured with MG63.2 OS cells. Polycarbonate 6-well Transwell® plates with 0.4 µm pores (#3412, Corning) were used for this experiment; MG63.2 OS cells were seeded in the Transwell, and TIIP cells were seeded in the bottom, both at a density of 5×10^4 cells/well. The cells were grown separately in their respective growth media for 48 H at 37°C and 5% CO₂. The cells were then combined, and the medium was replaced with a 1:1 mixture of TIIP media and MG63.2 media to initiate co-culture. After 5 days of incubation in co-culture, conditioned media supernatant (centrifugation at 12,000 x g for 10 minutes at 4°C) and total RNA were collected and stored at -80°C until use. TIIPs and MG63.2 OS cells that were grown separately/in monoculture served as controls.

RNA isolation and quantification

RNA was isolated from cells in the co-culture system as well as monoculture conditions using a commercial column-based system (RNeasy Mini Kit; #74104, Qiagen) as per the manufacturer's instructions. RNA concentration and purity were assessed using the NanoDrop™ One Spectrophotometer (ThermoFisher Scientific); samples with a 260/280 nm absorbance ratio of 1.8-2.0 were considered acceptable for sequencing.

RNAseq analysis

RNA samples were pooled and sequenced on NovaSeq Xplus 1.5B using Illumina® Stranded mRNA Prep and paired-end sequencing (300 cycle kit and 151bp_x2) to reveal biological pathways that were significantly enriched in TIIPs in co-culture with MG63.2 OS cells versus TIIPs in monoculture. The samples have 124 to 195 million pass-filter reads with more than 95% of bases above the quality score of Q30. Reads of the samples were trimmed for adapters and low-quality bases using Cutadapt before alignment with the reference genome (hg38) and the annotated transcripts using STAR. The average mapping rate of all samples is 95 to 97%. Unique alignment is above 76%. Library complexity is measured in terms of unique fragments in the mapped reads using Picard's MarkDuplicate utility. The samples have 52–70% non-duplicate reads. In addition, the gene expression quantification analysis was performed for all samples using STAR/RSEM tools. The normalized raw count data and the sample grouping information were provided as input to the edgeR (v.4.8.2) software at default parameters [26]. All genes were ranked based on their log fold change in expression, as determined by edgeR. This ranked list of genes for each group was then used as input to the standard Gene Set Enrichment Analysis (GSEA) pipeline, implemented in the fgsea (v1.36.2) package [27] using default parameters, to assess the relative enrichment of hallmark pathways in each condition. The top upregulated pathways are listed by their Normalized Enrichment Score (NES) and ordered by p-value.

Cytokine analysis

Conditioned media supernatant (centrifugation at 12,000 x g for 10 minutes at 4°C) was collected from the co-culture and monoculture conditions before lysing cells for RNA extraction to assess cytokine levels using the human XL cytokine array (#ARY022B, R&D Systems) according to the manufacturer's instructions. Briefly, 500 µl conditioned media was applied to each array, and the signal was visualized on a ChemiDoc imaging system (Bio-Rad). Automated densitometry of paired spots was performed using QuickSpots software (Ideal Eyes Systems). Arrays were background-subtracted and normalized to the average of the reference spots. Differentially expressed cytokines were identified by statistical comparison between the co-culture and monoculture conditions. Pathway analysis of significantly altered cytokines was performed using the online Enrichr tool (<https://maayanlab.cloud/Enrichr/>) [28–30].

Transforming growth factor beta-1 (TGF-β1) ELISA

TGF-β1 levels in conditioned media from the TIIP-OS co-culture system and monoculture conditions were quantified using the Human Quantikine™ TGF-β1 Immunoassay (#DB100C, R&D Systems) according to the manufacturer-recommended protocol. Concentrations in pg/mL were interpolated from a standard curve using GraphPad Prism 10.5.

Statistical analysis

Statistical analysis was performed in Microsoft Excel, R 4.5.2, and/or GraphPad Prism 10.5. All data were expressed as mean $\pm$ standard deviation (SD) of at least three independent experiments. Statistical significance for the differences between pairs of conditions was analyzed by a two-sample t-test. The criterion for statistical significance was considered as a p-value less than or equal to 0.05 (*p < 0.05; ** p < 0.01; *** p < 0.001).

Results

TIIPs accumulate at the NT:T interface of OS pulmonary metastases

We analyzed spatial profiling data from human metastatic osteosarcoma specimens to evaluate the spatial distribution of TIP and TIIP in relation to the metastatic lesions (Fig. 1, A). In contrast to the TIP signature, which demonstrated pneumocytes throughout the non-tumor (NT) lung tissue, the TIIP signature demonstrates notably higher enrichment at the non-tumor:tumor (NT:T) interface. This pattern is conserved with single gene enrichment of SPFTC, a commonly used marker of TIIPs[31, 32] (**Supplementary Fig. 1**). To extend upon these findings, SPC IHC was performed on OS pulmonary metastases from canine patients and a spontaneous metastasis K7M2-pEGFP murine model. In canine specimens, increased numbers of SPC-positive TIIPs were observed at the NT:T interface compared to the surrounding non-tumor lung parenchyma (p = 0.0083; Fig. 1, B and C). Similar findings were also observed in the murine model (p = 1.67e-05; Fig. 1, E and F). Together, these cross-species findings demonstrate that TIIPs accumulate at the periphery of OS pulmonary metastases.

TIIPs promote OS cell proliferation

TIIPs are a component of the TME of OS pulmonary metastases and are increasingly recognized as a potential player in tumor development [12, 33]. To determine whether TIIPs alter OS cell proliferation, MG63.2 cells were exposed to either primary human TIIPs via co-culture or TIIPs-conditioned media. MG63.2 cell proliferation significantly increased in co-culture with TIIPs compared to the control, i.e., co-culture media (p = 0.0005; Fig. 2, A). IncuCyte confluence measurements corroborated this finding, demonstrating higher MG63.2 cell confluence over time in co-culture conditions with TIIPs relative to the co-culture media control (Fig. 2, B). Interestingly, MG63.2 cells cultured in TIIP-conditioned media also demonstrated significantly greater proliferation compared to cells cultured in unconditioned TIIP media (p = 0.0057, Fig. 2, C and D). These findings suggest that the TIIPs may mediate pro-tumorigenic activity in the OS pulmonary metastatic niche.

Profibrotic signaling dominates TIIP-OS crosstalk

To characterize the transcriptional response of TIIPs to OS cells, RNAseq analysis was performed on TIIPs co-cultured with MG63.2 OS cells and compared to TIIPs maintained in monoculture. Pathway enrichment analysis revealed significant upregulation of the p53 signaling pathway in TIIPs co-cultured

with OS cells ($p\text{-adj} = 8.2 \times 10^{-3}$, Fig. 3, A). This finding is consistent with the known role of p53 signaling in TIIPs as a mediator of alveolar stress response [34]. Other significantly enriched pathways also included those associated with epithelial stress response, like oxidative phosphorylation, UV response, and hypoxia [35–37]. To determine whether this stress response was associated with changes in the secretome or vice versa, cytokine analysis was performed. Significant differences in 8 cytokines between co-culture and monoculture conditions were observed (Fig. 3, B and C). Specifically, GDF-15, a member of the TGF- β superfamily [38], was significantly elevated in TIIP-OS co-culture compared to monoculture conditions. Similarly, Dkk-1 and VEGF, amongst others, were significantly increased in co-culture. Media-only control showed no detectable expression of these cytokines. Pathway analysis of cytokines that were significantly elevated in co-culture conditions showed an enrichment of fibrotic and wound-healing pathways, including TGF- β Smad signaling, suggesting a model of cytokine-mediated fibrotic niche remodeling driven by TIIP-OS crosstalk (Fig. 3, D). TGF- β 1 was also assessed as it is known to mediate fibrosis [39, 40]; we found it to be significantly higher in conditioned media from co-culture of TIIPs and MG63.2 OS cells compared to conditioned media from TIIPs in monoculture ($p = 0.018$, Fig. 3, E). Notably, TGF- β 1 secretion was the highest by MG63.2 OS cells grown in monoculture, suggesting that the tumor cells themselves are a major source of this cytokine.

Discussion

This study provides the first cross-species characterization of TIIP distribution in the TME of pulmonary OS metastases and offers mechanistic insight into the dialogue between TIIPs and OS cells. We demonstrate that TIIPs accumulate at the NT:T interface in human, canine, and murine OS lung metastases; that TIIPs promote OS cell proliferation; that OS cell-derived signals drive transcriptional reprogramming of TIIPs with activation of the p53 pathway; and that TIIP-OS co-culture produces a cytokine milieu enriched in pro-fibrotic mediators. Taken together, these findings suggest that TIIPs participate in the construction of a fibrotic, pro-tumorigenic metastatic niche and nominate the TIIP-OS crosstalk axis as a potential therapeutic target in pulmonary OS metastasis.

The preferential accumulation of TIIPs at the NT:T interface across all three species strongly implies a functionally significant epithelial response to tumor invasion. Whether this accumulation reflects directed TIIP migration toward tumor-derived chemotactic signals, local proliferative expansion driven by injury-repair signaling, or impaired clearance from the peri-tumoral zone remains to be determined. However, the cross-species conservation of this spatial pattern argues powerfully for its biological role at the metastatic front. TIIPs normally function as progenitor cells responsible for alveolar repair and as coordinators of innate immune response [41]. Increased TIIP concentration at the advancing tumor border raises the possibility that they co-opt their wound-repair and immune-modulatory programs to establish a microenvironment permissive for tumor outgrowth. This is consistent with emerging evidence that metastatic niches are not simply structural habitat for arriving cancer cells but are engineered through the functional repurposing of tissue-resident cell populations [42–44].

Studies utilizing TIIPs in an *in vitro* setting are necessary to deconvolute their interactions with tumor cells. However, research remains highly elusive since stable cell lines such as A549, H441, RLE-6N, L2 and MLE12/15 fail to resemble the defining transcriptional and functional properties of TIIPs. Moreover, the isolation of pure, viable, and proliferative TIIPs for functional studies is fraught with challenges, particularly due to the difficulty in maintaining the well-differentiated TIIP phenotype of their *in vivo* counterpart [45, 46]. In this study, we utilized commercially available TIIPs that demonstrated comparable gene expression to patient-derived pneumocytes in culture [47, 48]. MG63.2 osteosarcoma cells that had been conditioned in the lung microenvironment of a nude mouse were co-cultured with TIIPs and demonstrated a significant increase in OS cell proliferation. The growth-permissive effect on MG63.2 OS cells was also achieved with exposure to TIIP conditioned media. These data suggest a model in which TIIPs at the tumor periphery possibly secrete a cocktail of growth-promoting factors that create a tumor-supportive environment. Rodrigues et al. have proposed the concept of “reflected stemness” where cancer cells prime TIIPs and TIIPs in turn promote stem-like features in cancer cells, enhancing tumorigenic programs in the lung tissue by creating a positive feedback loop [48].

The p53 pathway enrichment observed in TIIPs co-cultured with OS cells is mechanistically informative, as the p53 pathway is well established as a mediator of TIIP response to alveolar stress and injury. This is particularly relevant in idiopathic pulmonary fibrosis (IPF), a chronic disease characterized by TIIP pathology, in which p53-dependent senescence generates a sustained pro-fibrotic secretome including TGF- β 1, IL-6, and other mediators that drive myofibroblast activation and irreversible lung remodeling [49–51]. The activation of an analogous transcriptional program in TIIPs co-cultured with OS cells suggests that OS cells may exploit conserved epithelial stress-response pathways to generate a fibrotic environment. Fibrosis has been associated with enhanced tumor cell survival, immunosuppression, and therapy resistance across multiple cancer types. Indeed, recent studies have noted a fibrotic encapsulation surrounding OS pulmonary metastases that has been linked to poor responses to immunotherapy, potentially through the exclusion of cytotoxic T cells from the tumor interior [23, 52]. The molecular overlap between OS-educated TIIPs and IPF-associated TIIP biology raises the possibility that this fibrotic encapsulation is at least partially driven by TIIP reprogramming [41].

The cytokine profile of TIIP-OS co-culture supernatants, including significantly increased production of GDF-15, Dkk-1, and VEGF, provides further mechanistic insight. GDF-15, a member of the TGF- β superfamily, is significantly elevated in co-culture and has established roles in stress response and fibrosis in the lung parenchyma [53, 54]. p53 regulates the transcription of GDF-15, promoting epithelial-mesenchymal transition (EMT) in TIIPs and myofibroblast differentiation via a TGF- β receptor [55, 56]. Dkk-1, a Wnt signaling antagonist which also plays a role in fibrosis, is known to be expressed in OS and has been implicated in metastatic niche remodeling [57, 58]. The role of VEGF in pulmonary fibrosis is paradoxical, acting as both an anti-fibrotic protector and a pro-fibrotic promoter depending on the context [59]. Its anti-fibrotic role was proposed by Hamada et al., who showed that VEGF-targeted gene therapy attenuated fibrosis [60]. Today, nintedanib is an FDA-approved drug for the treatment of IPF that targets VEGFR alongside FGFR and PDGFR [61, 62]. The positive correlation between VEGFA expression and pulmonary metastasis in human OS patients further contextualizes this finding. In human OS

tumors, the VEGFA level was positively correlated with the presence of lung metastasis [63]. Pathway enrichment analysis of differentially expressed cytokines using Enrichr confirmed enrichment of wound healing and fibrotic processes, including TGF β Smad signaling, supporting the concept of a cytokine-mediated fibrotic remodeling of the alveolar niche driven by secretions from TIIPs and OS cells [64].

Abundant TGF- β induces excessive deposition of ECM via differentiation of fibroblasts into myofibroblasts, subsequent overproduction of collagen and fibers, and inhibition of collagen degradation, resulting in irreversible scar formation/wound healing, pulmonary fibrosis, and loss of lung function [65–67]. Although we could not attribute TGF- β 1 production to TIIPs in our co-culture system, studies have shown exogenous TGF- β stimulates profibrotic activity in TIIPs *in vitro*, such as, EMT, myofibroblast formation, and collagen synthesis [68, 69]. We found that OS cells themselves secrete high levels of TGF- β 1, positioning them as a plausible source of the signal that drives TIIP-mediated fibrotic activity in our co-culture system. This finding aligns with reports of active TGF- β signaling in primary OS tumors as well as OS pulmonary metastases [70, 71]. More broadly, OS-derived TGF- β has been shown to exert wide-ranging effects on the lung TME: inducing EMT, inhibiting antitumor immune responses, and enhancing localized fibrosis as well as angiogenesis [72]. These observations suggest tumor-secreted TGF- β may contribute to stromal remodeling in the metastatic lung niche, with TIIPs as one potential responsive cell population. Our *in vitro* co-culture model, while mechanistically informative, does not fully recapitulate the complexity of the *in vivo* lung TME, which includes dynamic interactions among immune cells, fibroblasts, endothelial cells, and the ECM. This does not invalidate the conclusions drawn here, but it underscores the importance of validating key findings in more physiologically representative model systems [3].

The convergence of our findings with the molecular pathobiology of IPF has important therapeutic implications. Anti-fibrotic agents including nintedanib and pirfenidone target TGF- β 1 signaling and downstream fibrotic effectors and have been FDA-approved for IPF [73]. This raises the hypothesis that repurposing anti-fibrotic therapies to disrupt TIIP-mediated niche remodeling could limit metastatic progression in OS. Yiu et al. have shown that both pirfenidone and nintedanib suppressed pulmonary metastasis in an LM8-Dunn murine model of OS pulmonary metastasis [52]. Likewise, Roberts' group has shown that nintedanib effectively reduced metastatic tumor burden in murine models of OS pulmonary metastases [74]. TGF- β antagonists have been shown to play an anti-tumor role in OS by suppressing proliferation as well as metastatic progression [75–77]. In addition, the combination of a TGF- β inhibitor and a PD-1/PD-L1 inhibitor, a bisphosphonate, a RANKL inhibitor, or cytotoxic chemotherapies could improve efficacy [78]. Many phase 1/2 clinical trials have shown TGF- β antagonists are safe and well-tolerated as treatment for other cancers [79, 80]. Thus, utilizing the naturally occurring canine OS model, with its intact immune system and clinically relevant metastatic disease course, provides an ideal platform to validate such interventions using the Comparative Oncology Trials Consortium clinical trial infrastructure to meet the therapeutic urgency for metastatic osteosarcoma [81].

Conclusion

This study demonstrates that TIIPs are a consistent and active component of the OS pulmonary metastatic microenvironment across species. TIIPs accumulate at the NT:T interface, promote OS cell proliferation *in vitro*, undergo p53 pathway-driven transcriptional reprogramming in the presence of OS cells, and together adopt a pro-fibrotic secretory phenotype. These findings suggest that there may be an overlap between the TIIP response in OS and IPF, expanding our understanding of the functionality of TIIPs in the metastatic niche. TIIPs represent a previously under-recognized host cell population with a crucial role in niche remodeling, and targeting TIIP-mediated pro-tumorigenic/fibrotic signaling represents a compelling new therapeutic hypothesis for limiting pulmonary metastatic progression of OS in both human and canine patients.

Declarations

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References

1. Lee JA, Lim J, Jin HY et al (2021) Osteosarcoma in adolescents and young adults. Cells 10. <https://doi.org/10.3390/cells10102684>
2. Edmunds GL, Smalley MJ, Beck S et al (2021) Dog breeds and body conformations with predisposition to osteosarcoma in the UK: a case-control study. Canine Med Genet 8. <https://doi.org/10.1186/s40575-021-00100-7>
3. Beck J, Ren L, Huang S et al (2022) Canine and murine models of osteosarcoma. Vet Pathol 59:399–414

4. Meltzer PS, Helman LJ (2021) New Horizons in the Treatment of Osteosarcoma. *N Engl J Med* 385:2066–2076. <https://doi.org/10.1056/nejmra2103423>
5. Silver KI, Patkar S, Mazcko C et al (2023) Patterns of metastatic progression and association with clinical outcomes in canine osteosarcoma: A necropsy study of 83 dogs. *Vet Comp Oncol* 21:646–655. <https://doi.org/10.1111/vco.12927>
6. Geller DS, Gorlick R (2010) Osteosarcoma: a review of diagnosis, management, and treatment strategies. *Clin Adv Hematol Oncol* 8:705–718
7. Smeland S, Bielack SS, Whelan J et al (2019) Survival and prognosis with osteosarcoma: outcomes in more than 2000 patients in the EURAMOS-1 (European and American Osteosarcoma Study) cohort. *Eur J Cancer* 109:36–50. <https://doi.org/10.1016/j.ejca.2018.11.027>
8. Bielack SS, Kempf-Bielack B, Delling G et al (2002) Prognostic Factors in High-Grade Osteosarcoma of the Extremities or Trunk: An Analysis of 1,702 Patients Treated on Neoadjuvant Cooperative Osteosarcoma Study Group Protocols. *J Clin Oncol* 20:776–790. <https://doi.org/10.1200/JCO.2002.20.3.776>
9. Wilk SS, Zabielska-Koczywas KA (2021) Molecular mechanisms of canine osteosarcoma metastasis. *Int J Mol Sci* 22
10. Roy J, Wycislo KL, Pondenis H et al (2017) Comparative proteomic investigation of metastatic and non-metastatic osteosarcoma cells of human and canine origin. *PLoS ONE* 12. <https://doi.org/10.1371/journal.pone.0183930>
11. Paoloni M, Davis S, Lana S et al (2009) Canine tumor cross-species genomics uncovers targets linked to osteosarcoma progression. *BMC Genomics* 10:625. <https://doi.org/10.1186/1471-2164-10-625>
12. McGee LE, Pereira JS, McEachron TA et al (2025) The tumor microenvironment of metastatic osteosarcoma in the human and canine lung. *Commun Biol* 8
13. Reinecke JB, Jimenez Garcia L, Saraf AJ et al (2025) Metastasis-Initiating Osteosarcoma Subpopulations Establish Paracrine Interactions with Lung and Tumor Cells to Create a Metastatic Niche. *Cancer Res* 85:4341–4358. <https://doi.org/10.1158/0008-5472.CAN-24-3360>
14. Yang C, Tian Y, Zhao F et al (2020) Bone microenvironment and osteosarcoma metastasis. *Int J Mol Sci* 21:1–17. <https://doi.org/10.3390/ijms21196985>
15. Du X, Wei H, Zhang B et al (2023) Molecular mechanisms of osteosarcoma metastasis and possible treatment opportunities. *Front Oncol* 13
16. Xu Wting, Bian Z, yu, Fan Q et al (2009) ming, Human mesenchymal stem cells (hMSCs) target osteosarcoma and promote its growth and pulmonary metastasis. *Cancer Lett* 281:32–41. <https://doi.org/10.1016/j.canlet.2009.02.022>
17. Luo C, Min X, Zhang D (2024) New insights into the mechanisms of the immune microenvironment and immunotherapy in osteosarcoma. *Front Immunol* 15
18. Christenson JL, Williams MM, Richer JK (2022) The underappreciated role of resident epithelial cell populations in metastatic progression: contributions of the lung alveolar epithelium. *Am J Physiol*

19. Castranova V, Rabovsky J, Tucker JH, Miles PR (1988) The Alveolar Type II Epithelial Cell. A Multifunctional Pneumocyte
20. Corbière V, Dirix V, Norrenberg S et al (2011) Phenotypic characteristics of human type II alveolar epithelial cells suitable for antigen presentation to T lymphocytes. *Respir Res* 12. <https://doi.org/10.1186/1465-9921-12-15>
21. Montagner M, Bhome R, Hooper S et al (2020) Crosstalk with lung epithelial cells regulates Sfrp2-mediated latency in breast cancer dissemination. *Nat Cell Biol* 22:289–296
22. Zhou Y, Liu Q, Dai X et al (2018) Transdifferentiation of type II alveolar epithelial cells induces reactivation of dormant tumor cells by enhancing TGF- β 1/SNAI2 signaling. *Oncol Rep* 39:1874–1882. <https://doi.org/10.3892/or.2018.6247>
23. Eigenbrood J, Wong N, Mallory P et al (2025) Spatial Profiling Identifies Regionally Distinct Microenvironments and Targetable Immunosuppressive Mechanisms in Pediatric Osteosarcoma Pulmonary Metastases. *Cancer Res* 85:2320–2337. <https://doi.org/10.1158/0008-5472.CAN-24-3723>
24. LeBlanc AK, Mazcko CN, Cherukuri A et al (2021) Adjuvant sirolimus does not improve outcome in pet dogs receiving standard-of-care therapy for appendicular osteosarcoma: A prospective, randomized trial of 324 dogs. *Clin Cancer Res* 27:3005–3016
25. Su Y, Luo X, He BC et al (2009) Establishment and characterization of a new highly metastatic human osteosarcoma cell line. *Clin Exp Metastasis* 26:599–610. <https://doi.org/10.1007/s10585-009-9259-6>
26. Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinf Appl NOTE* 26:139–140. <https://doi.org/10.1093/bioinformatics/btp616>
27. Korotkevich G, Sukhov V, Budin N et al (2021) Fast gene set enrichment analysis. <https://doi.org/10.1101/060012>. bioRxiv 060012
28. Chen EY, Tan CM, Kou Y et al (2013) Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics* 14. <https://doi.org/10.1186/1471-2105-14-128>
29. Kuleshov MV, Jones MR, Rouillard AD et al (2016) Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res* 44:W90–W97. <https://doi.org/10.1093/NAR/GKW377>
30. Xie Z, Bailey A, Kuleshov MV et al (2021) Gene Set Knowledge Discovery with Enrichr. <https://doi.org/10.1002/cpz1.90>
31. Lee DF, Salguero FJ, Grainger D et al (2018) Isolation and characterisation of alveolar type II pneumocytes from adult bovine lung. *Sci Rep* 8. <https://doi.org/10.1038/s41598-018-30234-x>
32. Jain KG, Liu Y, Zhao R et al (2024) Surfactant Protein-C Regulates Alveolar Type 2 Epithelial Cell Lineages via the CD74 Receptor. *J respiratory biology translational Med* 1:10017. <https://doi.org/10.70322/JRBTM.2024.10017>

33. Dacheux MA, Lee SC, Shin Y et al (2022) Prometastatic Effect of ATX Derived from Alveolar Type II Pneumocytes and B16-F10 Melanoma Cells. *Cancers (Basel)* 14:.
<https://doi.org/10.3390/cancers14061586>
34. Juan-Guardela BM, Herazo-Maya JD (2026) Targeting epithelial stress and inflammation through p53: therapeutic implications for pulmonary fibrosis. *Am J Respir Crit Care Med* 212:1187–1188.
<https://doi.org/10.1093/AJRCCM/AAMAG121>
35. Lottes RG, Newton DA, Spyropoulos DD, Baatz JE (2014) Alveolar type II cells maintain bioenergetic homeostasis in hypoxia through metabolic and molecular adaptation. *Am J Physiol Lung Cell Mol Physiol* 306:L947. <https://doi.org/10.1152/AJPLUNG.00298.2013>
36. Zhou Y, Hill C, Yao L et al (2021) Quantitative Proteomic Analysis in Alveolar Type II Cells Reveals the Different Capacities of RAS and TGF- β to Induce Epithelial–Mesenchymal Transition. *Front Mol Biosci* 8:595712. <https://doi.org/10.3389/FMOLB.2021.595712>
37. Zeng L, Yan J (2025) Mechanisms of alveolar type II epithelial cells' mitochondrial quality control during acute lung injury/acute respiratory distress syndrome: bridging the gap between oxidative stress, inflammation, and fibrosis. *Front Physiol* 16:1684729.
<https://doi.org/10.3389/FPHYS.2025.1684729>
38. Al-Mudares F, Reddick S, Ren J et al (2020) Role of Growth Differentiation Factor 15 in Lung Disease and Senescence: Potential Role Across the Lifespan. *Front Med (Lausanne)* 7:594137.
<https://doi.org/10.3389/FMED.2020.594137>
39. Meng X, Nikolic-Paterson DJ, Yao Lan H (2016) TGF- β : the master regulator of fibrosis.
<https://doi.org/10.1038/nrneph.2016.48>
40. Kanamori M, Sato I, Koo CX et al (2026) Selective blockade of latent TGF- β 1 activation suppresses tissue fibrosis with good safety. *Communications Medicine* 2026 6:1 6:131-.
<https://doi.org/10.1038/s43856-026-01408-w>
41. Ruaro B, Salton F, Braga L et al (2021) The history and mystery of alveolar epithelial type ii cells: Focus on their physiologic and pathologic role in lung. *Int J Mol Sci* 22:1–16
42. Xiao G, Wang X, Xu Z et al (2025) Lung-specific metastasis: the coevolution of tumor cells and lung microenvironment. *Mol Cancer* 24
43. de Visser KE, Joyce JA (2023) The evolving tumor microenvironment: From cancer initiation to metastatic outgrowth. *Cancer Cell* 41:374–403
44. Xiao G, Wang X, Xu Z et al (2025) Lung-specific metastasis: the coevolution of tumor cells and lung microenvironment. *Mol Cancer* 24
45. Beers MF, Moodley Y (2017) When Is an alveolar type 2 cell an alveolar type 2 cell? A conundrum for lung stem cell biology and regenerative medicine. *Am J Respir Cell Mol Biol* 57:18–27
46. Dobbs LG (1990) Isolation and culture of alveolar type II cells.
<https://doi.org/10.1152/ajplung19902584L134> 258:. <https://doi.org/10.1152/AJPLUNG.1990.258.4.L134>

47. Alysandratos KD, Garcia-De-Alba C, Yao C et al (2023) Culture impact on the transcriptomic programs of primary and iPSC-derived human alveolar type 2 cells. *JCI Insight* 8.
<https://doi.org/10.1172/jci.insight.158937>
48. Rodrigues FS, Karoutas A, Ruhland S et al (2024) Bidirectional activation of stem-like programs between metastatic cancer and alveolar type 2 cells within the niche. *Dev Cell* 59:2398–2413e8.
<https://doi.org/10.1016/j.devcel.2024.05.020>
49. Dupont S, Zacchigna L, Adorno M et al (2004) Convergence of p53 and TGF-beta signaling networks. *Cancer Lett* 213:129–138
50. Higgins SP, Tang Y, Higgins CE et al (2018) TGF- β 1/p53 signaling in renal fibrogenesis. *Cell Signal* 43:1–10
51. Parimon T, Yao C, Stripp BR et al (2020) Alveolar epithelial type II cells as drivers of lung fibrosis in idiopathic pulmonary fibrosis. *Int J Mol Sci* 21. <https://doi.org/10.3390/ijms21072269>
52. Yui Y, Kumai J, Watanabe K et al (2022) Lung fibrosis is a novel therapeutic target to suppress lung metastasis of osteosarcoma. *Int J Cancer* 151:739–751. <https://doi.org/10.1002/IJC.34008>
53. GDF15 is an epithelial- derived biomarker of idiopathic pulmonary fibrosis.
<https://journals.physiology.org/doi/epdf/10.1152/ajplung.00062.2019>. Accessed 22 Jul 2026
54. Takenouchi Y, Kitakaze K, Tsuboi K, Okamoto Y (2020) Growth differentiation factor 15 facilitates lung fibrosis by activating macrophages and fibroblasts. *Exp Cell Res* 391.
<https://doi.org/10.1016/J.YEXCR.2020.112010>
55. Zhang Y, Jiang M, Nouraie M et al (2019) GDF15 is an epithelial-derived biomarker of idiopathic pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 317:L510.
<https://doi.org/10.1152/AJPLUNG.00062.2019>
56. Lian W, Cheng D, Sun W et al (2025) Senescent alveolar type II epithelial cells-secreted GDF15 promotes silicosis progression via interfering intercellular communication. *Ecotoxicol Environ Saf* 292:117917. <https://doi.org/10.1016/J.ECOENV.2025.117917>
57. Lee N, Smolarz AJ, Olson S et al (2007) A potential role for Dkk-1 in the pathogenesis of osteosarcoma predicts novel diagnostic and treatment strategies. *Br J Cancer* 97:1552–1559.
<https://doi.org/10.1038/sj.bjc.6604069>
58. Klavdianou K, Liossis SN, Daoussis D (2017) Dkk1: A key molecule in joint remodelling and fibrosis. *Mediterr J Rheumatol* 28:174. <https://doi.org/10.31138/MJR.28.4.174>
59. Murray LA, Habiel DM, Hohmann M et al (2017) Antifibrotic role of vascular endothelial growth factor in pulmonary fibrosis. *JCI Insight*. <https://doi.org/10.1172/JCI.INSIGHT.92192>. 2:
60. Hamada N, Kuwano K, Yamada M et al (2005) Anti-vascular endothelial growth factor gene therapy attenuates lung injury and fibrosis in mice. *J Immunol* 175:1224–1231.
<https://doi.org/10.4049/JIMMUNOL.175.2.1224>
61. Chaudhary NI, Roth GJ, Hilberg F et al (2007) Inhibition of PDGF, VEGF and FGF signalling attenuates fibrosis. *Eur Respir J* 29:976–985. <https://doi.org/10.1183/09031936.00152106>

62. Richeldi L, du Bois RM, Raghu G et al (2014) Efficacy and Safety of Nintedanib in Idiopathic Pulmonary Fibrosis. *N Engl J Med* 370:2071–2082.
https://doi.org/10.1056/NEJMOA1402584/SUPPL_FILE./NEJMOA1402584_DISCLOSURES.PDF
63. Kaya M, Wada T, Akatsuka T et al (2000) Vascular Endothelial Growth Factor Expression in Untreated Osteosarcoma Is Predictive of Pulmonary Metastasis and Poor Prognosis. *Clin Cancer Res* 6:572–577
64. Margadant C, Sonnenberg A (2010) Integrin-TGF- β crosstalk in fibrosis, cancer and wound healing. *EMBO Rep* 11:97–105. <https://doi.org/10.1038/EMBOR.2009.276/FIGURES/5>
65. Yue X, Shan B, Lasky JA (2010) TGF- β : Titan of Lung Fibrogenesis. <https://doi.org/10.2174/10067>
66. Verrecchia F, Mauviel A (2007) Transforming growth factor- β and fibrosis. *World J Gastroenterology: WJG* 13:3056. <https://doi.org/10.3748/WJG.V13.I22.3056>
67. Broekelmann TJ, Limper AH, Colby TV, McDonald JA (1991) Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proc Natl Acad Sci U S A* 88:6642. <https://doi.org/10.1073/PNAS.88.15.6642>
68. Enomoto Y, Katsura H, Fujimura T et al (2023) Autocrine TGF- β -positive feedback in profibrotic AT2-lineage cells plays a crucial role in non-inflammatory lung fibrogenesis. *Nat Commun* 14.
<https://doi.org/10.1038/s41467-023-40617-y>
69. Goldmann T, Zissel G, Watz H et al (2018) Human alveolar epithelial cells type II are capable of TGF β -dependent epithelial-mesenchymal-transition and collagen-synthesis. *Respir Res* 19.
<https://doi.org/10.1186/s12931-018-0841-9>
70. Expression of transforming growth factor β isoforms in osteosarcoma variants: association of tgfb β 1 with high-grade osteosarcomas.
<https://pathsocjournals.onlinelibrary.wiley.com/doi/epdf/10.1002/%28SICI%291096-9896%28199807%29185%3A3%3C284%3A%3AAID-PATH94%3E3.0.CO%3B2-Z>. Accessed 22 Jul 2026
71. Ge R, Huang GM (2023) Targeting transforming growth factor beta signaling in metastatic osteosarcoma. *J Bone Oncol* 43. <https://doi.org/10.1016/J.JBO.2023.100513>
72. P Grünwald TG, Mora J, Sant Joan de Déu, Barcelona H et al (2018) Transforming Growth Factor- β Signaling Plays a Pivotal Role in the Interplay Between Osteosarcoma Cells and Their Microenvironment. *Front Oncol* 8:133. <https://doi.org/10.3389/FONC.2018.00133>
73. Kou M, Jiao Y, Li Z et al (2024) Real-world safety and effectiveness of pirfenidone and nintedanib in the treatment of idiopathic pulmonary fibrosis: a systematic review and meta-analysis. *Eur J Clin Pharmacol* 80:1445–1460. <https://doi.org/10.1007/S00228-024-03720-7>
74. Reinecke JB, Garcia LJ, Gross AC et al (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>
75. LY2109761 inhibits metastasis and enhances chemosensitivity in osteosarcoma MG-63 cells.
<https://www.europeanreview.org/article/8736>. Accessed 23 Jun 2026

76. HE D, GAO J, ZHENG L et al (2021) TGF- β inhibitor RepSox suppresses osteosarcoma via the JNK/Smad3 signaling pathway. *Int J Oncol* 59. <https://doi.org/10.3892/IJO.2021.5264>
77. Choi SH, Myers JT, Tomchuck SL et al (2024) Oral transforming growth factor-beta receptor 1 inhibitor vactosertib promotes osteosarcoma regression by targeting tumor proliferation and enhancing anti-tumor immunity. *Cancer Commun* 44:884–888.
<https://doi.org/10.1002/CAC2.12589/ASSET/AEA50972-FAEE-41B2-B54B-F1DDC767AF46/ASSETS/GRAPHIC/CAC212589-FIG-0001.PNG>
78. Ge R, Huang GM (2023) Targeting transforming growth factor beta signaling in metastatic osteosarcoma. *J Bone Oncol*. 43
79. Huang CY, Chung CL, Hu TH et al (2021) Recent progress in TGF- β inhibitors for cancer therapy. *Biomed Pharmacother* 134:111046. <https://doi.org/10.1016/J.BIOPHA.2020.111046>
80. Johansen AM, Forsythe SD, Mcgrath CT et al TGF β in Pancreas and Colorectal Cancer: Opportunities to Overcome Therapeutic Resistance. <https://doi.org/10.1158/1078-0432.CCR-24-0468>
81. Paoloni M, Lana S, Thamm D et al (2010) The creation of the Comparative Oncology Trials Consortium Pharmacodynamic Core: Infrastructure for a virtual laboratory. *Vet J* 185:88–89.
<https://doi.org/10.1016/j.tvjl.2010.04.019>

Figures

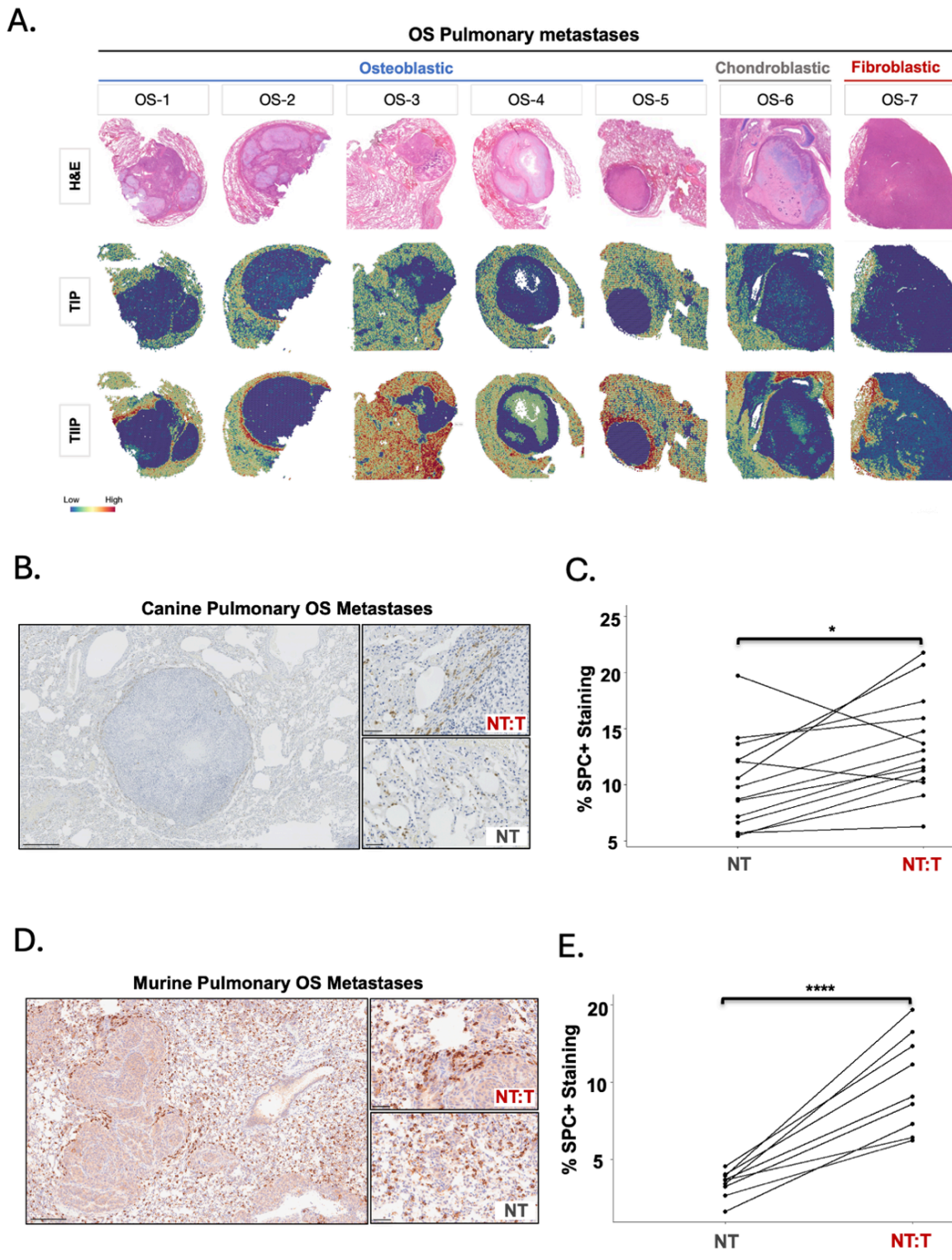


Figure 1

TIIPs accumulate at the peripheral margin of OS pulmonary metastases across species. (A) Spatial mapping of TIP and TIIP gene signatures in human OS pulmonary metastases. H&E-stained images for reference. **(B)** Representative IHC-stained images of canine pulmonary metastases stained for SPC. **(C)** Graphical representation of percentage positive SPC staining in the NT:T (non-tumor:tumor) versus NT (non-tumor) lung of canine OS metastases (n=14). **(D)** Representative IHC-stained images of murine

pulmonary metastases stained for SPC. **(E)** Graphical representation of percentage positive SPC staining in the NT:T (non-tumor:tumor) versus NT (non-tumor) lung of murine OS metastases (n=9). * $p \leq 0.05$, *** $p \leq 0.001$ (two-sample t-test).

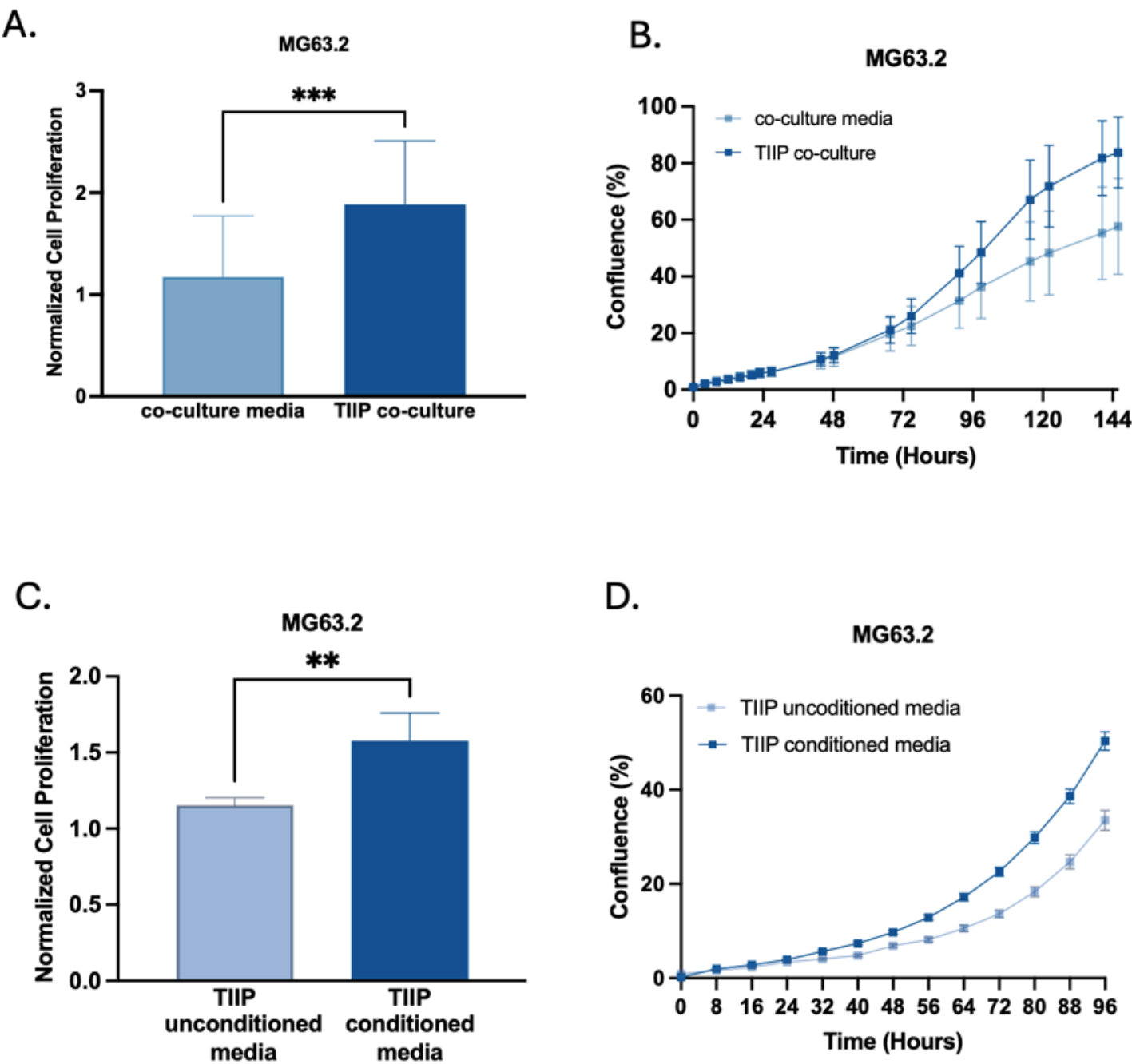


Figure 2

TIIPs promote OS cell proliferation. **(A)** Normalized cell proliferation of MG63.2 OS cells in co-culture with TIIPs and co-culture growth media at 72 H. Data represent the mean $\pm$ SD from three biological replicates; *** $p \leq 0.001$ (two-sample t-test). **(B)** IncuCyte phase-contrast percentile confluence over time (in hours) of MG63.2 OS cells in co-culture with TIIPs and co-culture growth media. **(C)** Normalized cell proliferation of MG63.2 OS cells in TIIP conditioned media and unconditioned media at 72 H. **(D)**

IncuCyte phase-contrast percentile confluence over time (in hours) of MG63.2 OS cells in TIIP conditioned media and unconditioned media.

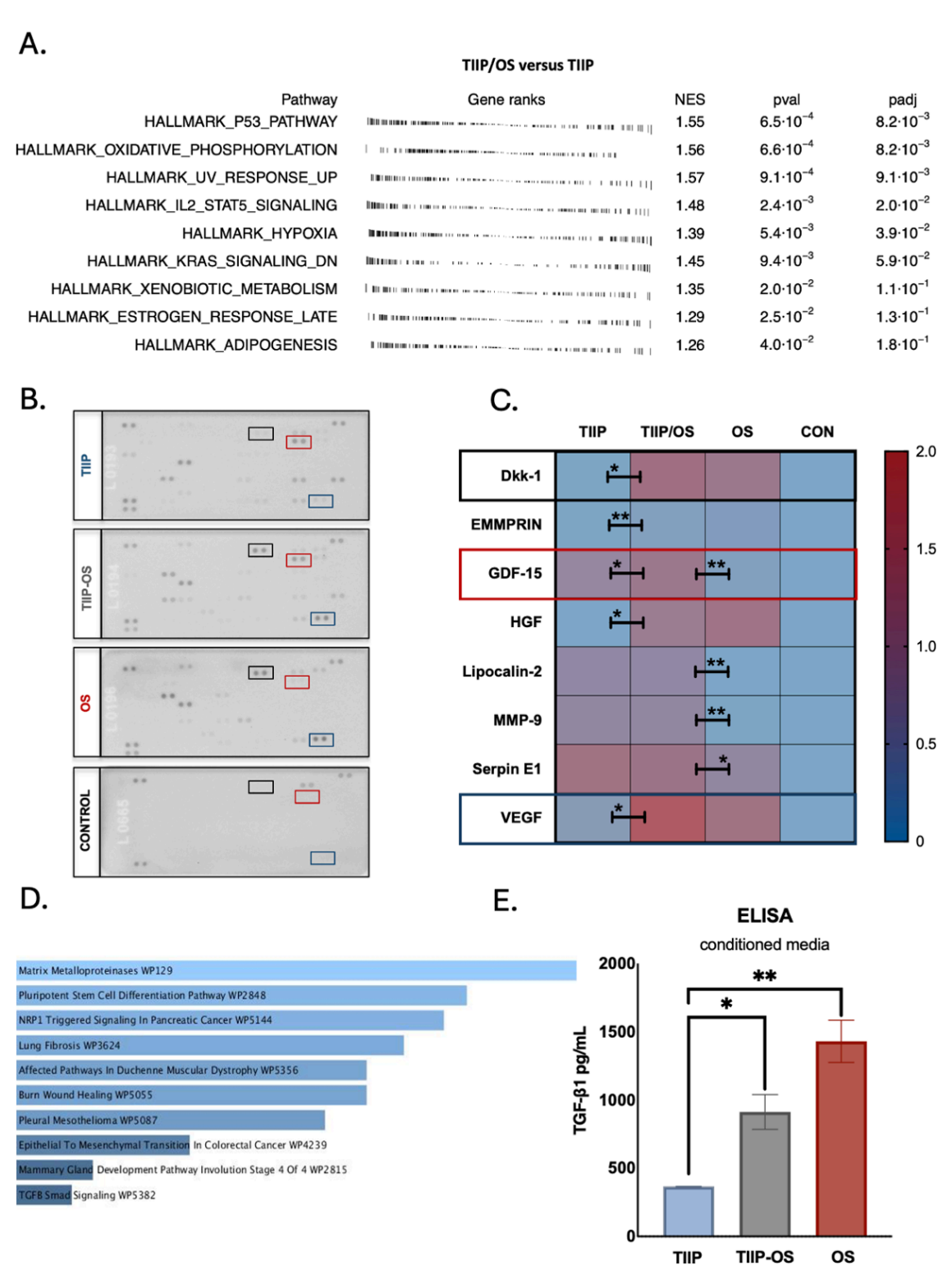


Figure 3

Profibrotic signaling dominates TIIP-OS crosstalk. (A) Top pathways that are significantly enriched in TIIPs in co-culture versus monoculture, listed by their Normalized Enrichment Score (NES) and ordered

by p-value. **(B)** Representative images of cytokine arrays exposed to conditioned media collected from co-culture and monoculture of TIIPs and OS cells. **(C)** Heatmap showing normalized densitometry of significantly different cytokines in co-culture versus monoculture conditions. Uncultured media served as the control. Data represent the mean $\pm$ SD from three biological replicates; * $p \leq 0.05$, ** $p \leq 0.01$ (two-sample t-test). **(D)** Enrichr pathway analysis of significantly elevated cytokines in co-culture ranked by p-value. **(E)** TGF- β 1 concentrations (pg/mL) in conditioned media collected from co-culture and monoculture of TIIPs and OS cells. Data represent the mean $\pm$ SD from three biological replicates; * $p \leq 0.05$, ** $p \leq 0.01$ (two-sample t-test).

Supplementary Files

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