Fibroblast activation protein-α (FAP) as an immunotherapy biomarker and CAR T-cell target in the osteosarcoma tumor microenvironment.

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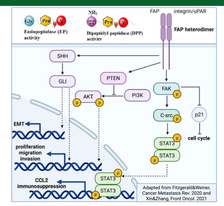
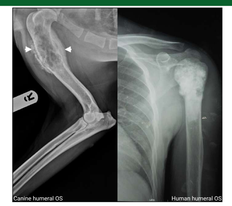
BACKGROUND





- In more common carcinoma cancers, fibroblast activation protein-α (FAP) has been explored as a(n):
  - Prognostic indicator
  - Cancer-associated fibroblast (CAF) marker
  - PET radionuclide target for metastatic lesion imaging
  - Target for anti-CAF CAR T cell therapy
- Emerging studies document FAP expression in osteosarcoma (OS) and some soft tissue sarcomas (STS) but the functional, clinical, and therapeutic significance of FAP expression by tumor and stromal cells in the sarcoma tumor microenvironment (TME) is unknown
- FAP has both enzymatic and non-enzymatic reverse signaling activity, and may serve distinct functions when expressed in sarcoma cells vs. CAFs or in primary vs. metastatic settings
- Canine OS and STS develop spontaneously within an intact immunologic context and closely parallel human OS in clinical, molecular, and immunologic presentation
  - Dogs with OS are an animal model and patient population uniquely suited to studying FAP in the native sarcoma TME

We hypothesize that FAP expression in OS cells and/or CAFs may predict TME composition and allow for co-targeting of OS cells and tumor-supporting CAFs with CAR T cell therapy.

FAP IS EXPRESSED BY CANINE SARCOMA CELLS AND STROMA

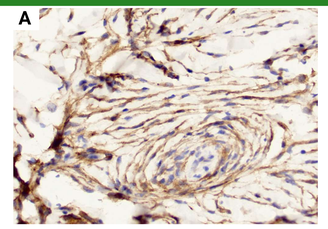


Figure 2 Tumor-type and cellular distribution of FAP expression in canine primary STS and metastatic OS. (A) FAP immunolabeling in tumor/stromal cells within a canine grade II STS. **(B)** FAP expression is highest in the most common presentations of canine STS with low grade tumors and peripheral nerve sheath tumors (PNST) showing significantly higher expression compared with high grade tumors and canine fibrosarcomas (FSA). **(C)** FAP immunolabeling in tumor and stromal cells at the invasive front of a canine OS lung metastasis. **(D)** OS cells and lung CAFs have the highest FAP expression among OS TME cells by scRNAseq of canine OS lung metastases.

FAP-HIGH OS TUMORS HAVE A MORE IMMUNOSUPPRESSIVE TME

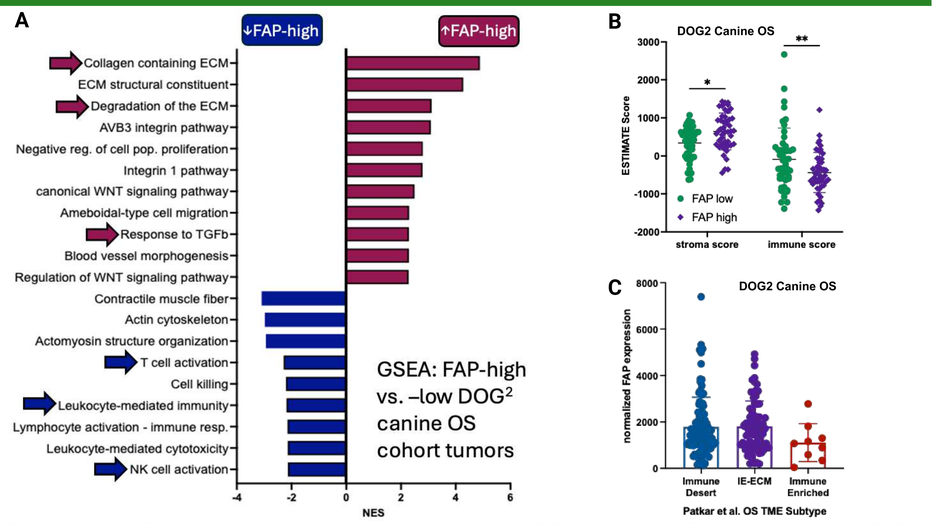


Figure 4 Transcriptomic profiling of canine OS indicates an association between high FAP expression and a fibrotic, immune-excluded TME. **(A)** Functional enrichment analysis of differentially expressed genes between FAP-high and FAP-low canine OS tumors from the DOG[2] Canine OS cohort reveals notable upregulation of matrix remodeling, angiogenic, and WNT signaling pathways and downregulation of effector immune-related transcriptional programs in FAP-high tumors. **(B)** Tumor compartment deconvolution using ESTIMATE methodology (Yoshihara et al. *Nat Commun.* 2013) supports increased stromal and decreased immune components in FAP-high DOG[2] OS tumors. **(C)** OS TME subtypes defined by Patkar et al. (*Clin Cancer Res* 2024) with the poorest prognosis (immune desert, immune enriched dense extracellular matrix-like) also have the highest FAP expression levels within the DOG[2] canine OS cohort.

KEY METHODS

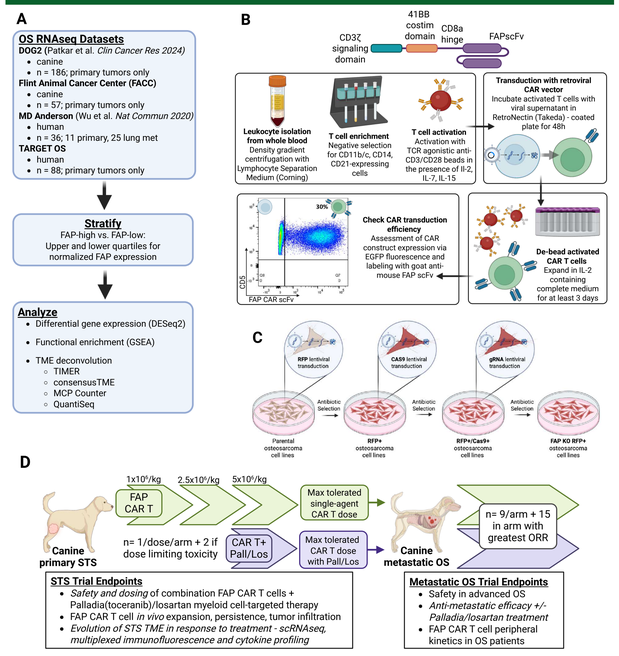


Figure 1 Overview of key methods for evaluating FAP as a CAR T cell therapy target and biomarker of TME composition in OS. (A) Description and sources for OS transcriptional datasets and analysis workflow. **(B)** Schematic of a second-generation human, canine, and mouse cross-reactive FAP CAR construct and associated canine CAR T cell manufacturing protocol. **(C)** Protocol for making FAP CRISPR knockout OS cell lines. **(D)** Trial design and key endpoints for phase 1 and 2 clinical trials of combination FAP CAR T cell and Palladia/losartan therapy in dogs with spontaneously occurring STS and OS.

FAP-TARGETING CANINE CAR T CELLS KILL OS CELLS & CAFs

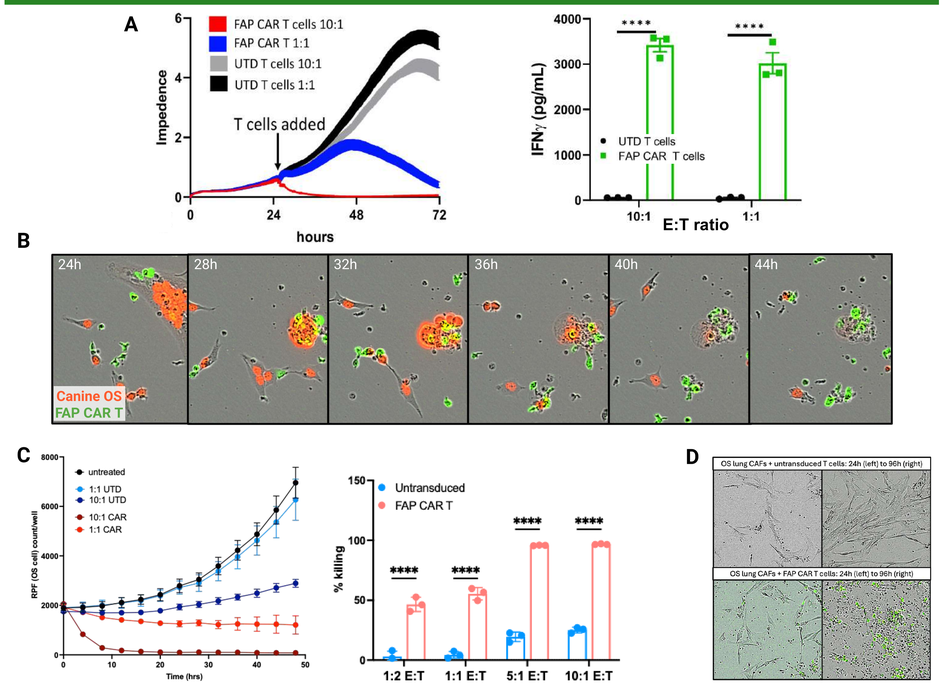


Figure 3 *in vitro* activity of canine FAP-targeting CAR T cells. (A) FAP CAR T cells generated from healthy canine donor T cells demonstrate CAR-dependent *in vitro* killing and effector cytokine release in response to FAP-expressing canine OS cells. **(B)** Representative live-cell fluorescence images of OS cell killing by FAP CAR T cells over time. **(C)** FAP CAR T cells generated from canine donors with primary OS show similar robust activity against FAP-expressing canine OS cells as those produced from healthy dog donors. **(D)** Representative live-cell fluorescence images of FAP CAR T cells killing OS canine OS lung CAFs over time.

FAP EXPRESSION IS ASSOCIATED WITH RECRUITMENT OF SUPPRESSIVE MYELOID CELL POPULATIONS IN HUMAN OS

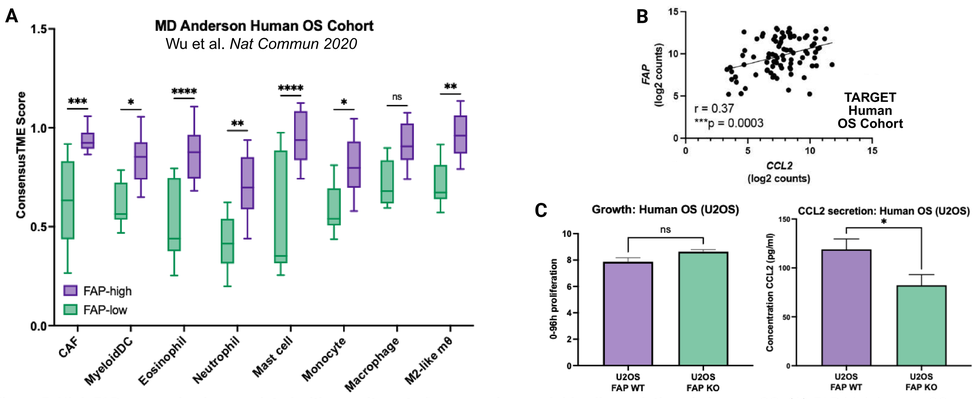
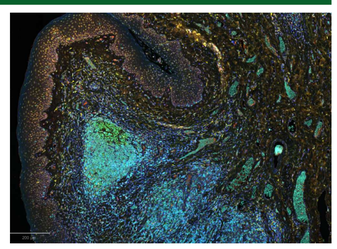


Figure 5 High FAP expression is associated with recruitment of suppressive myeloid cell populations in human OS. (A) FAP-high human OS samples from the MD Anderson OS cohort (Wu et al. *Nat Commun* 2020) show enrichment of CAF and myeloid cell immunotranscriptomic signatures supporting a fibrotic, myeloid-enriched OS TME associated with FAP expression. Cell type deconvolution was performed on bulk mRNA expression data using ConsensusTME methodology and cell type gene signatures (Jiménez-Sánchez et al. Cancer Res 2019). **(B)** FAP expression correlates with increased expression of the prognostic myeloid-recruiting chemokine CCL2 in human OS tumors from the TARGET OS cohort. **(C)** CRISPR knockout of FAP in the human OS cell line U2OS does not alter cell proliferation but significantly reduces CCL2 secretion.

CONCLUSIONS, ONGOING WORK & FUTURE PLANS

- **FAP is expressed by tumor cells and CAFs in OS tumors, allowing for targeted killing of these important TME cell types with FAP CAR T cells**
  - **A first-in-dog clinical trial assessing the safety, kinetics, and TME modifying activity of FAP CAR T cells alone or in combination with Palladia/losartan myeloid cell-depleting therapy is underway**
- **FAP expression by OS cells and/or CAFs may play a functional role in cultivating an immunosuppressive or immune-excluded OS TME**
- Experiments evaluating growth, migration, and cytokine secretion in additional human and canine FAP KO OS cell lines are also ongoing
- Future work will evaluate FAP expression and immune infiltrates in **canine and human OS** lung metastases via multispectral immunofluorescence (mIF) to:
  - localize FAP expression to OS cells vs. stroma
  - correlate tumor vs. stromal FAP with immune infiltrates and outcome



mIF image of canine tonsil tissue labeled with a panel of canine and cross-reactive antibodies optimized by our laboratory for identifying and phenotyping major tumor-infiltrating immune cell subsets. CD3 T cells (aqua), PAX5 B cells (green), CD204 macrophages (yellow), Myc (orange), FOXP3 Tregs (red), DAPI cell nuclei (navy).

ACKNOWLEDGEMENTS

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* p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001 by two-tailed T-test (2 groups), ANOVA (>2 groups), or Spearman correlation