

Introduction and Rationale

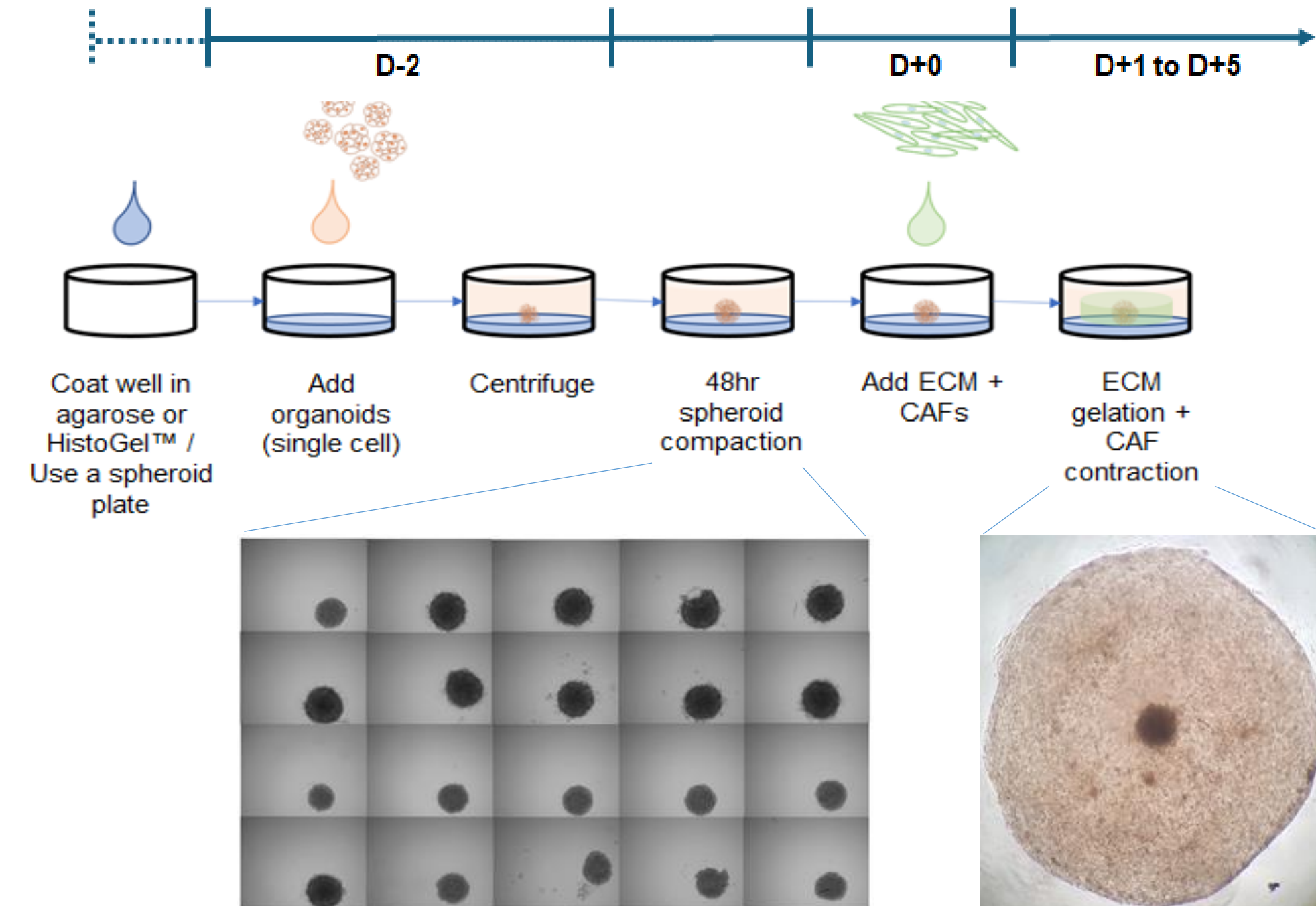
- Cancer-associated fibroblasts (CAFs) are cells found in the tumor stroma and are emerging as mediators of treatment resistance.
- CAFs are a heterogeneous cell population; CAF subtypes with distinct transcriptomic profiles and spatial tissue distributions have been identified.
- It remains unclear which CAF subsets are responsible for the fundamental processes of tumor growth, invasion, and response to therapy.
- This study aims to establish a novel *in vitro* model that recapitulates the *in vivo* tumor-stroma spatial relationship to investigate CAF heterogeneity and its impact on tumor growth in non-small cell lung cancer (NSCLC).

Methods

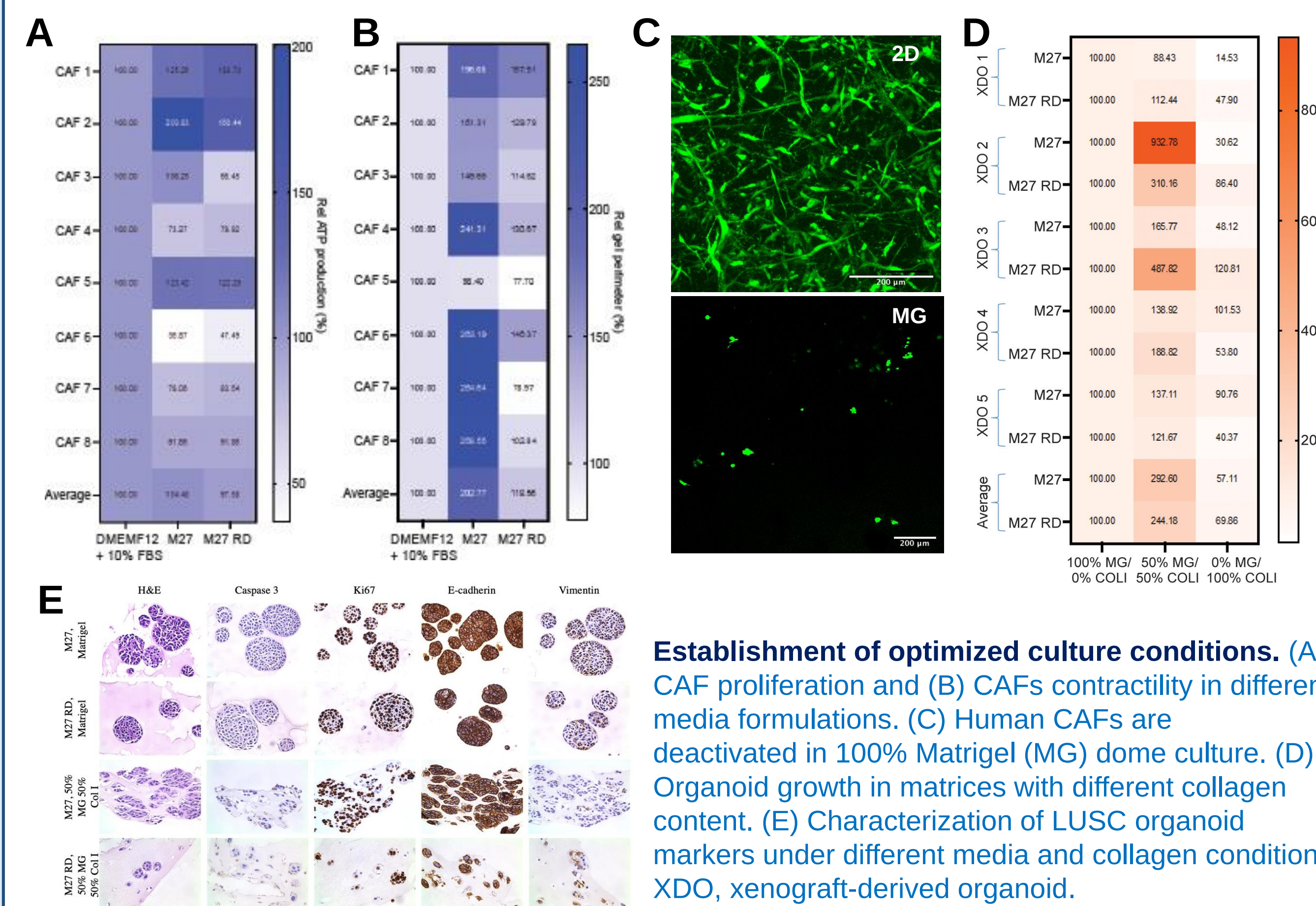
Assembly of 3D- *in vitro* model

I. Co-culture workflow:

- Co-cultures (CCs) are established between NSCLC tumor organoids and NSCLC primary CAFs.
- The co-culture model allows for 3D cellular interactions in a spatially controlled manner.
- The co-culture model is compatible with live imaging, IHC/IF, dissociation, and downstream assays.



II. Optimization and harmonization of culture conditions:



Establishment of optimized culture conditions. (A) CAF proliferation and (B) CAFs contractility in different media formulations. (C) Human CAFs are deactivated in 100% Matrigel (MG) dome culture. (D) Organoid growth in matrices with different collagen content. (E) Characterization of LUSC organoid markers under different media and collagen conditions XDO, xenograft-derived organoid.

References

- Pramod S. et al., Heterogeneity and plasticity of cancer-associated fibroblasts, Nature Cancer 2025.
- Lavie D. et al., Cancer-associated fibroblasts in the single-cell era, Nature Cancer 2022.
- Attieh Y. et al., Cancer-associated fibroblasts lead tumor invasion through integrin-b3-dependent fibronectin assembly. J. Cell Biol. 2017

Development of a histology-inspired NSCLC organoid-CAF model

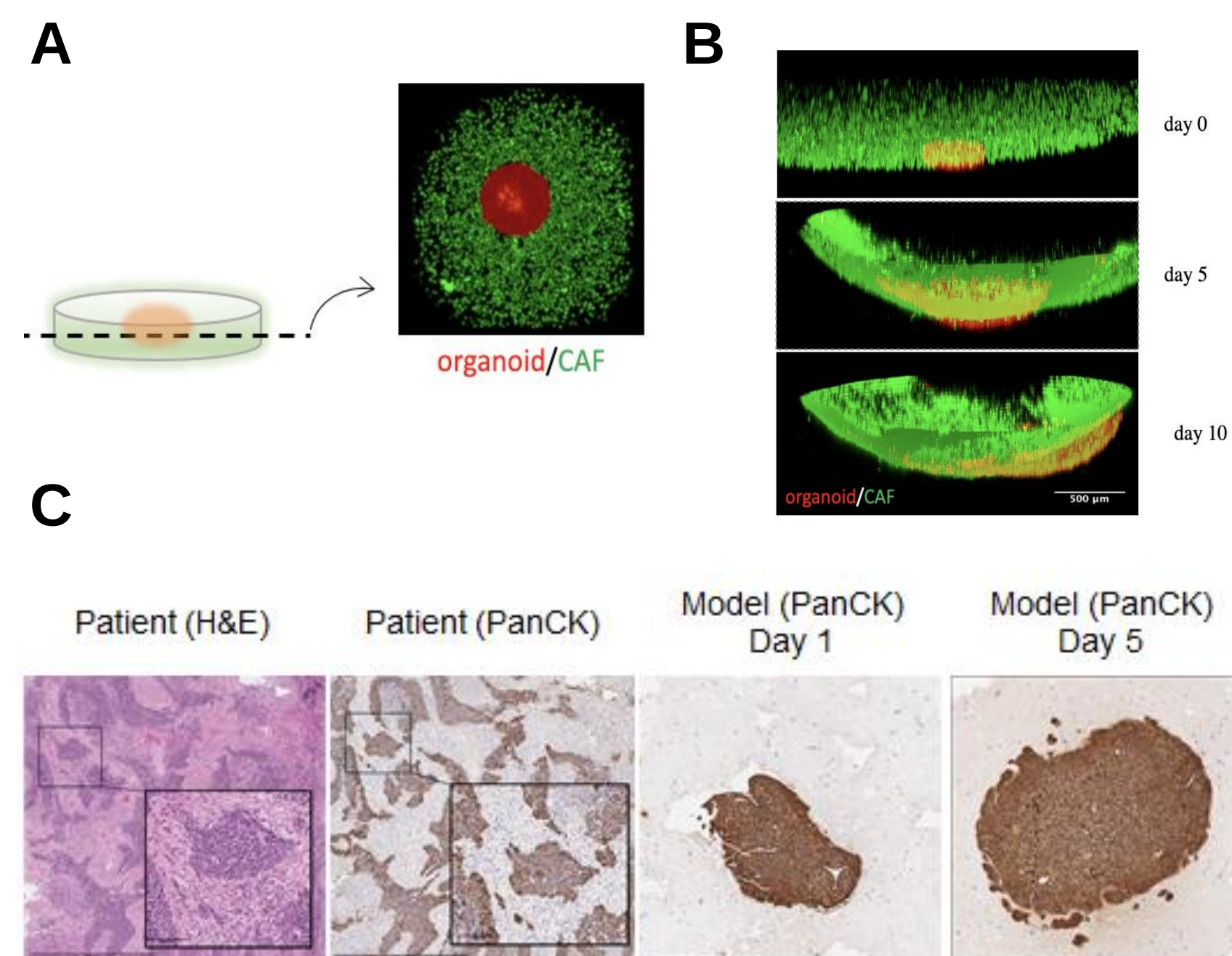


Fig. 1. Assembly of histology-inspired NSCLC *in vitro* model. Confocal images showing tumor organoid spheroids (red) and CAFs (green), (A) Top and (B) lateral views of spheroid-CAF co-cultures over time. (C) Morphological comparison of PanCK- stained patient LUSC tumor islands and the *in vitro* model.

Functional studies on CAF heterogeneity

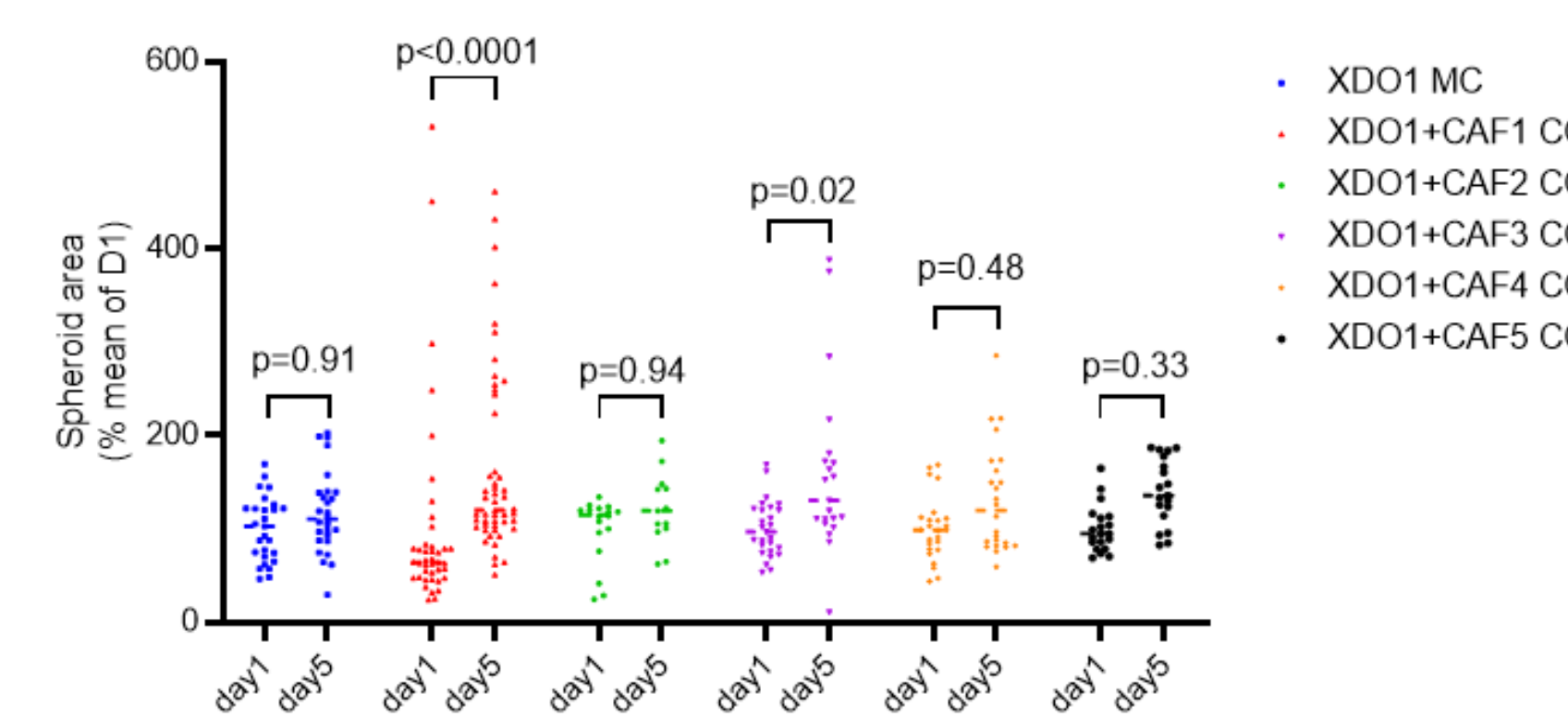


Fig. 2. Expansion of LUSC tumor cells in co-culture with CAFs. Total LUSC tumor spheroid area from day 1 to day 5 was quantified using imageJ/Fiji software.

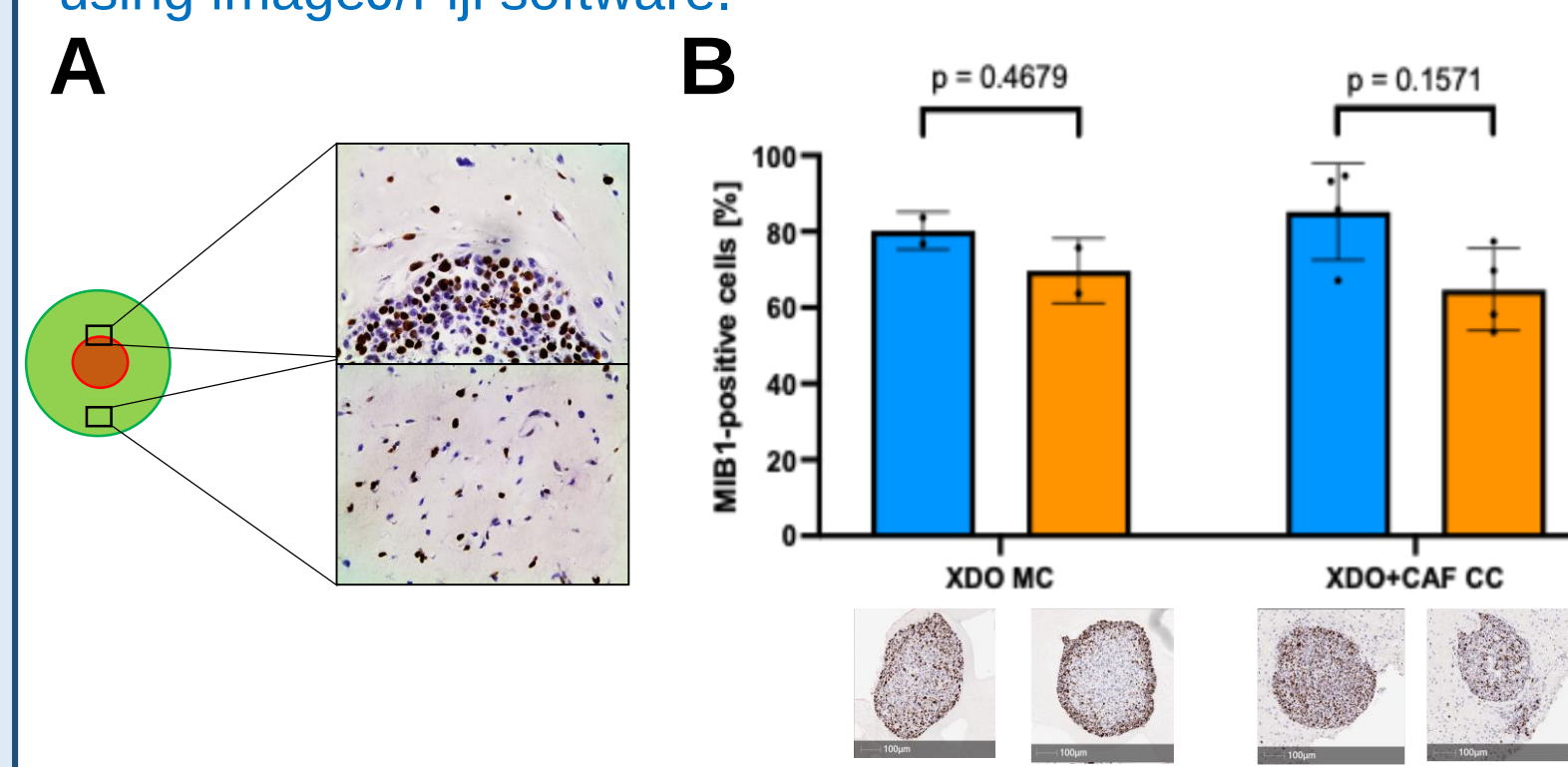


Fig. 3. CAF effect on tumor cell spreading is independent of proliferation. (A) Proliferative cells (tumor cells and CAFs) at the interface between compartments and within the stroma on day 5 of co-culture. (B) Quantification on MIB-1 staining demonstrates that the CAF effect on tumor cell spreading is not due to increased tumor cell proliferation.

Results

CAF subtypes produce distinct spatial invasion patterns

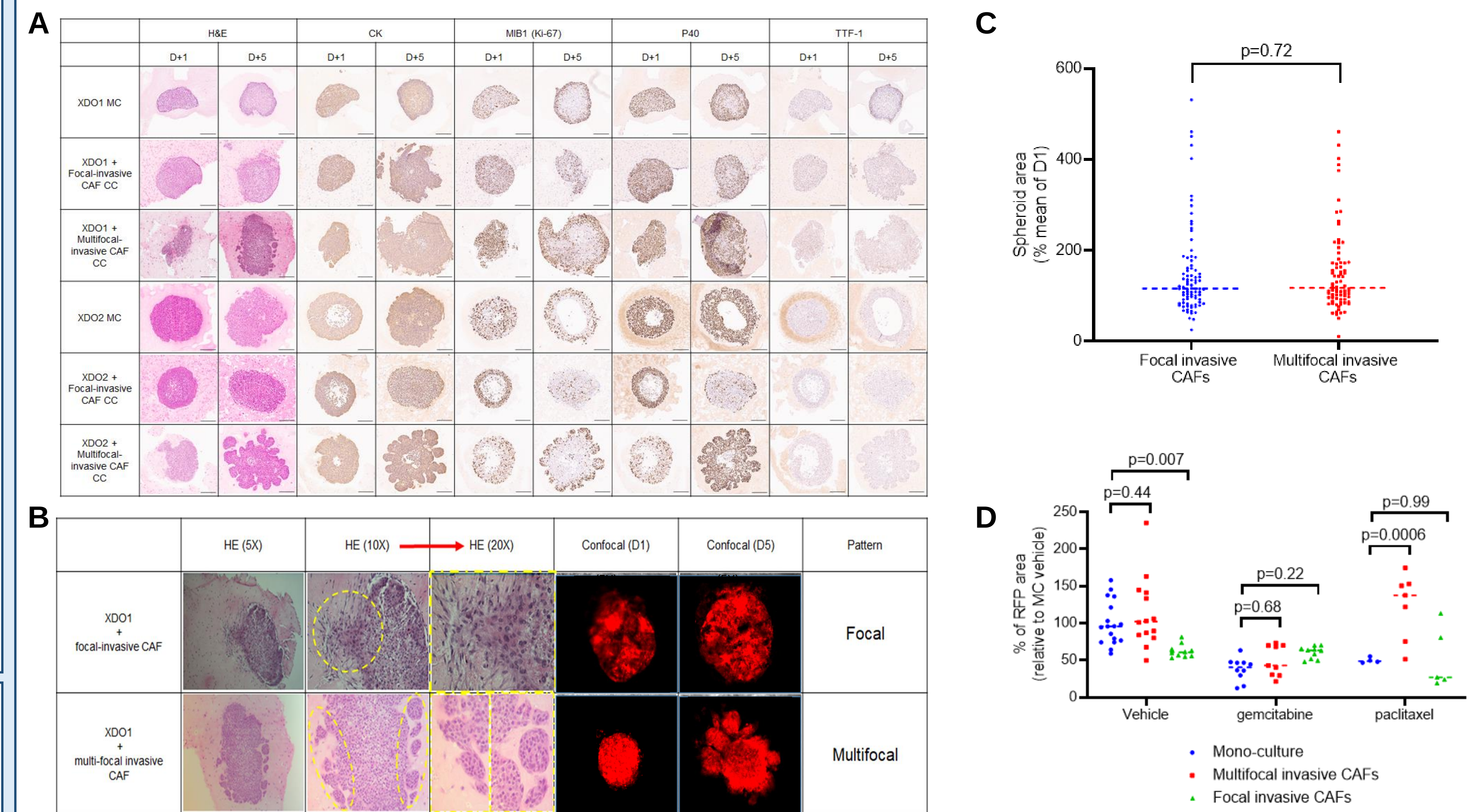


Fig. 4. Characterization of invasive CAFs. *In vitro* co-culture model showing two distinct types of invasive CAF. (A) *In vitro* mono-culture (MC) and co-culture (CC) models characterized by immunohistochemistry (IHC). (B) Representative images showing focal and multifocal invasive effect of CAFs on tumor cells by H&E staining and confocal microscopy. (C) Total spheroid area was quantified using imageJ/Fiji in all co-cultures with focal invasive CAFs and multifocal invasive CAFs. (D) The percentage of RFP-labeled spheroid area was quantified using ImageJ/Fiji following treatment with gemcitabine or paclitaxel in mono-culture and co-culture with focal invasive CAFs and multifocal invasive CAFs.

Gene expression validation of co-culture model

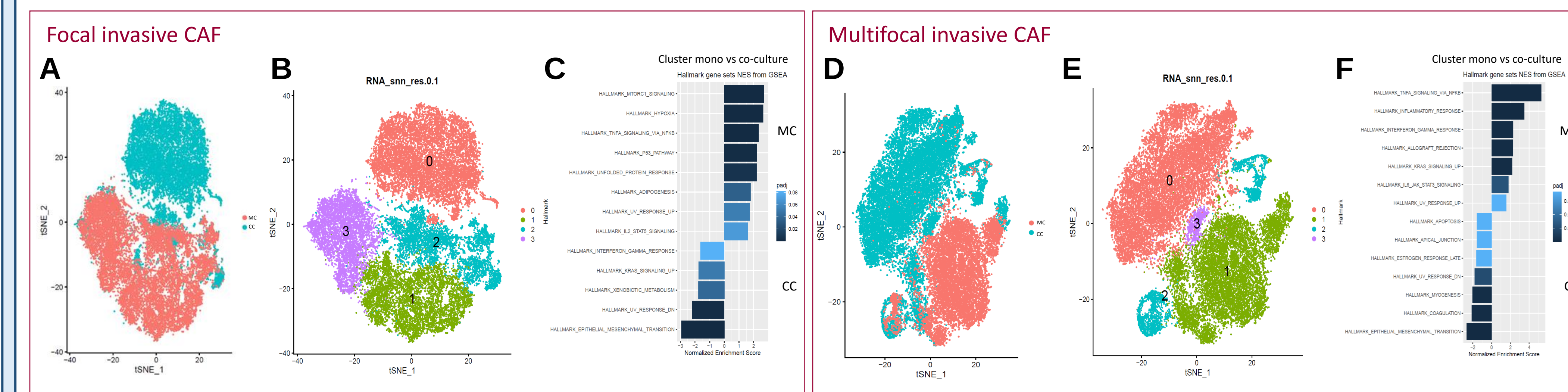


Fig. 5. Single-cell RNA sequencing reveals cellular heterogeneity of CAFs between mono-culture and co-culture models. scRNA-seq analysis identifies significant differentially expressed genes (DEGs) between mono-culture (MC) and co-culture (CC) CAFs. GSEA identifies CAF signature genes associated with EMT. (A, D) tSNE plots showing cell-type annotations for MC and CC CAFs. (B, E) Seurat clustering of two distinct LUSC CAFs. (C, F) GSEA bar plot showing enriched pathways associated with up- and down-regulated DEGs in cluster 1 compared with cluster 0 based on scRNA-seq data.

Conclusion

- Establishing harmonized culture conditions for NSCLC organoid-CAF co-cultures enables the characterization of cellular phenotypes.
- Histology-informed model architecture provides a valuable platform for studying tumor-stroma communication.
- CAFs exhibit functional heterogeneity, defining focal versus multifocal tumor cell invasion and displaying distinct responses to chemotherapy treatment.
- scRNA-seq analysis identifies significant differentially expressed genes (DEGs) between mono-culture and co-culture CAFs, suggesting that both distinct CAF populations exhibit significant Epithelial-mesenchymal transition (EMT)-CAF-related genes in co-culture, which may contribute to tumor microenvironment remodeling and cancer progression.

Acknowledgements

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