# Clonal Dominance Defines Metastatic Dissemination in Pancreatic Cancer


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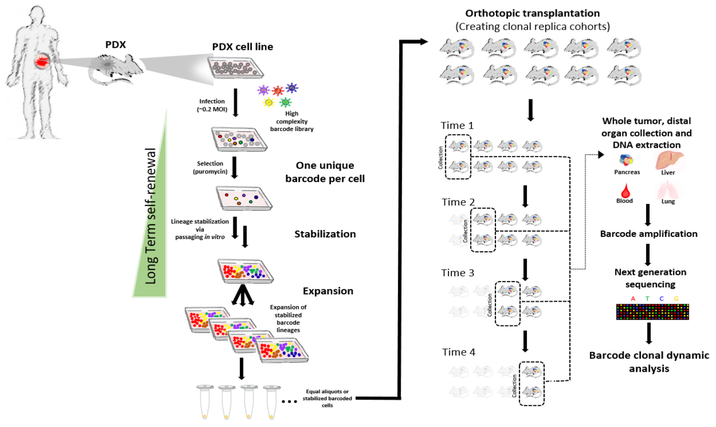
## Introduction

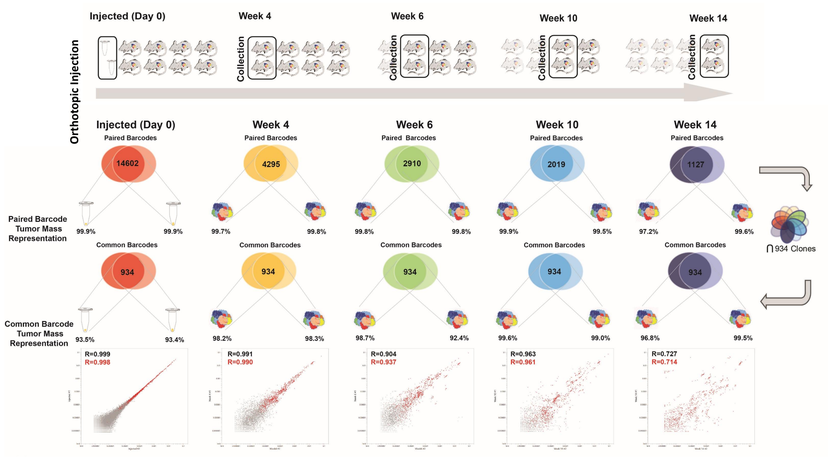
Tumors can be viewed as a complex ecosystem where multiple sub-clones harboring specific functional phenotype compete and co-evolve. The evolution and dynamics of individual clonal behavior in tumor has long been a topic of high interest, as it is essential to our understanding of how tumors progress, evolve and adapt to therapeutics. However, currently there are no robust models that allow the systematic monitoring of clonal dynamics overtime, as well as for paired functional characterization of individual sub-clones with specific behaviors of interest. Therefore, we established a high resolution longitudinal clonal tracing model that comprehensively capture lineage dynamics during tumor expansion and dissemination without perturbing the natural expansion of the tumor.

## Generation of Orthotopic Clonal Replica Tumors



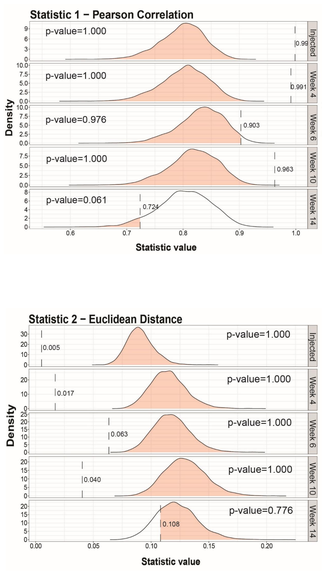

Patient derived xenograft cell lines from pancreatic cancer (primary tumor) are barcoded with a high complexity barcode library (~50M variations) at low MOI for 1 unique barcode per cell. Barcoded cells are then stabilized in vitro where clones with long-term self-renewal properties are naturally enriched. Post stabilization, cells are expanded and transplanted orthotopically in mouse cohorts. Primary tumor (pancreas) and distal organs are collected at various time points and sequenced for barcode information.

## Orthotopic Clonal Replica (oCRT) Tumors - Biological Reproducibility




Two mouse were sacrificed at each time point and their respective tumor clonal lineage composition and relative abundance is quantitatively analyzed from barcode readout via NGS. The number of paired barcodes shared between two biological replicates at the same time point and their relative tumor mass representation is shown in the upper Venn diagram. Each lineage's relative abundance (%, log scale) between two biological replicates are plotted as gray dots in X-Y scatter plots. The number of common barcodes shared across all time points and their relative tumor mass representation is shown in the lower Venn diagram. Each lineage's relative abundance (%, log scale) between two biological replicates are plotted as red dots in X-Y scatter plots.

## Statistical reproducibility Based on Parametric Bootstrap




Use statistically robustness test on the clonal (barcode) lineage composition amongst CRTs cohorts to inform us whether clonal replica tumors composition and intra-tumor lineages expansion were relatively similar across cohorts.

**Statistic 1**:
*T1* is the Pearson correlation coefficient between populations *Px* and *Py*. If T1 is close to 1, then A and B is similar.

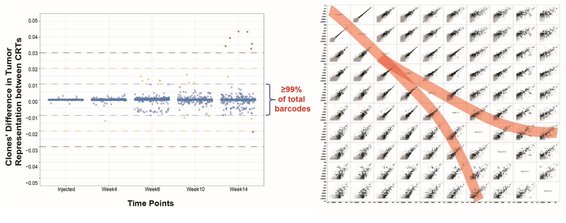
**Statistic 2**:
*T2* is the Euclidean distance between *Px* and *Py*,

$$T_2 = ||p_X - p_Y||_2 \ (d_{A,B} = \sqrt{\sum_{i=1}^{n=A\cup B}(p_A - p_B)^2})$$

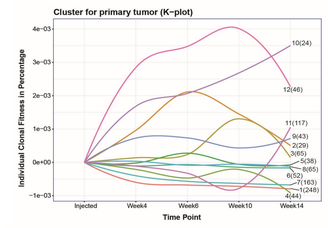
If T2 is close to 0, then A and B is similar.

**Simulation**: Bootstrap resampling approach to perform similarity test (5000X)
**Baseline criteria for similarity**: Based on differences between empirical data from the two injected vials (identical technical replicates) and two week 4 samples.

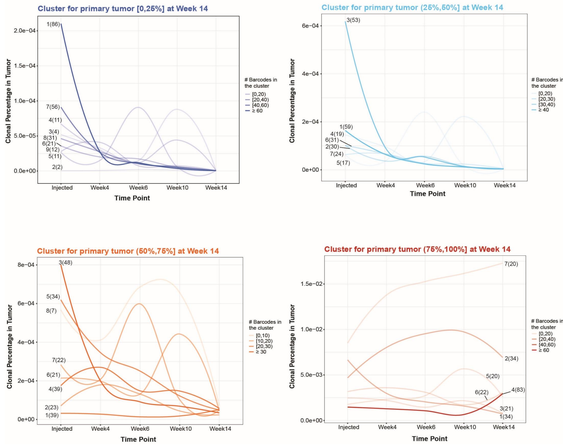
**Natural divergence of clones over time (stochastic aspect of tumor expansion)**




## Unperturbed Clonal Dynamics Reveal Alternating Clonal Dominance as a Distinct Features of Tumor Growth

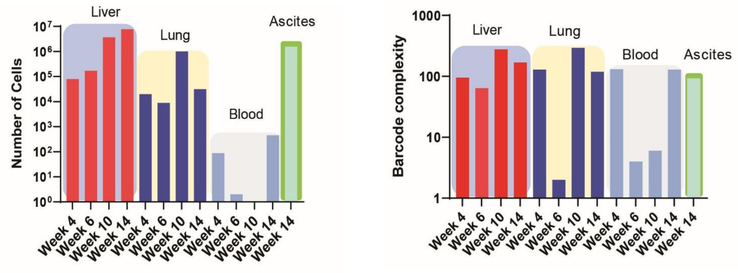



"K-plot" displaying longitudinal clonal dynamics in the primary tumor over 14 weeks. Each barcode is converted into % representation and normalized to the "injected" time point for each time point and connected. Each line represents a unique barcode's longitudinal dynamic in terms of individual clonal fitness in the tumor. Graph indicates multiple clonal evolution patterns (displaying 12 patterns) over time.

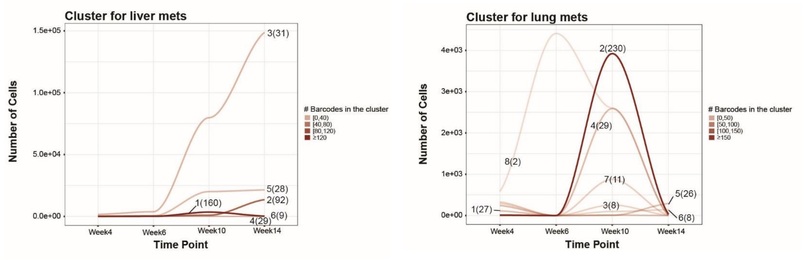



Longitudinal clonal dynamics in the primary tumor over 14 weeks by quartiles based on accumulative tumor mass at the last time point (week 14). Top to bottom panel display clusters of clonal lineages with similar behavior dynamics at quartile of 0% – 25%, 25% –50%, 50% – 75%, and 75% –100% of the week 14 tumor respectively. Each line represents a unique barcode's longitudinal dynamic in terms of % representation. Each cluster's X-axis indicates time of collection and Y-axis indicates % tumor representation of the barcode. The number outside the bracket indicates the cluster name, and the number within the bracket indicates the exact number of barcodes within the cluster (darker colors are associated with larger number of barcodes within the cluster).

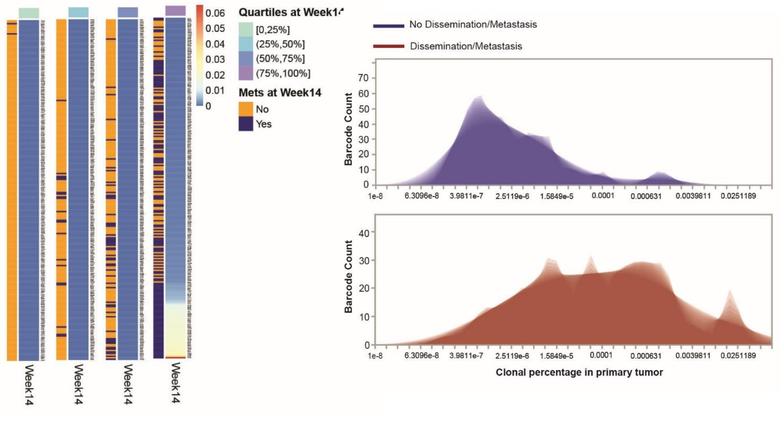
## Quantitative Assessment of Clonal Dissemination Dynamics Uncovers Clonal Dominance as a Major Determinant of Metastasis




(**Left**) Cumulative cell number at distant sites (liver, lung, blood and ascites) over time. Cell number at each time point is converted based on spike-in scales. (**Right**) Barcode complexity at distant sites over time.

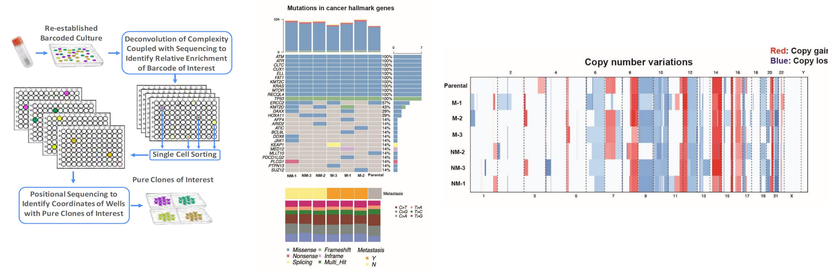



Dynamic subclonal growth in liver (**left**) and lung (**right**). Cell number at each time point is converted based on spike-in scales. Lineage behavior was grouped into discrete dynamic growth patterns (clusters) over time. The number outside the bracket indicates the cluster name, and the number within the bracket indciates the exact number of barcodes within the cluster (darker colors are associated with larger number of barcodes within the cluster).



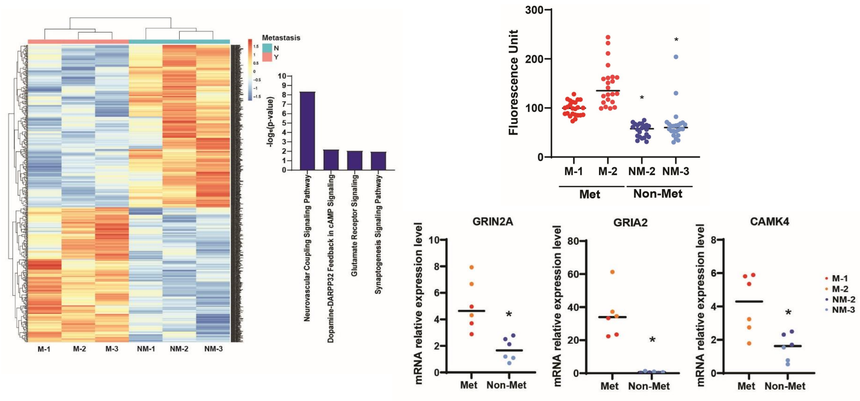

(**Left**) Heatmap of average subclonal abundance (percentage) in the primary tumors with dissemination/metastasis status (yes/no) at week 14. Dissemination of each subclone is indicated on the left of each heat map in quartiles (yellow, no dissemination; blue, dissemination). (**Right**) Average clonal abundance in the primary tumors (percentage) at week 14 for metastatic clones (top) and non-metastatic clones (bottom).

## Functional Characterization of Sub-clones via Clonal Isolation



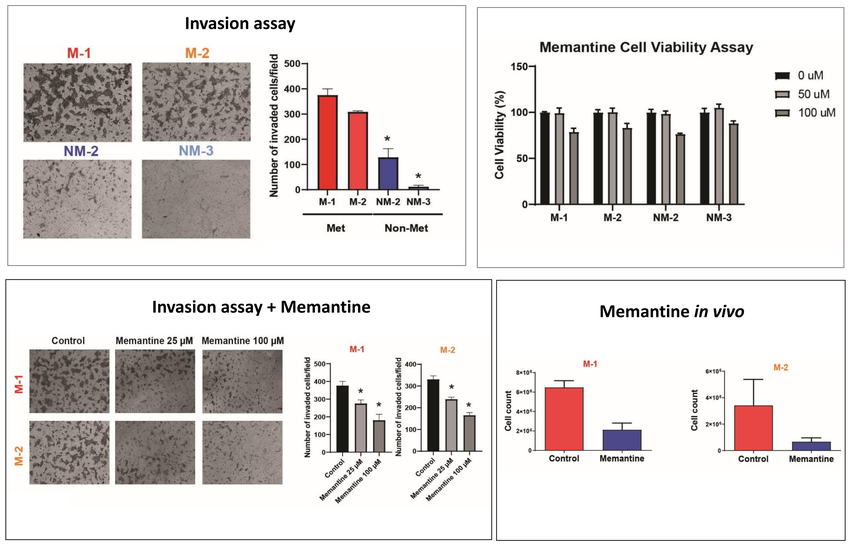

High-throughput clonal isolation workflow allows us to isolate and culture clones of interest (pro-met vs non-met) (**left**); Mutational landscape and CNV analysis of pro-met (M-1–3) and non-met (NM-1–3) clones from WES data (**mid and right**)

## Pro-Metastatic Clones Upregulation in NMDA Pathway



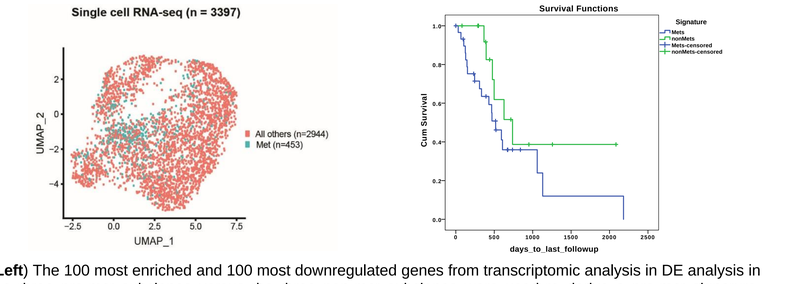

Heat map of differential gene expression of isogenic clones with distinct metastatic potential as determined in longitudinal oCRT studies (**left**). IPA analysis of pro-met (M-1–3) and non-met (NM-1–3) clones identifies enrichment of glutamate receptors and neurovascular coupling signaling pathways in pro-met lineages (**mid**). Basal intracellular calcium concentration of clonal lineages selected on the basis of differential metastatic behavior (**top right**). Validation of clonal DE in NMDA receptor, AMPA receptor, CaMK by qPCR (**bottom right**).

## NMDAR Antagonist Reduce Fitness and Invasion of Pro-Metastatic Clones




Representative images of invasion assay indicating the basal invasion potential of two pro-met and two non-met subclones (**top left**) and the effect of the non-competitive NMDA antagonist 25 or 100 μM memantine on invasive potential of cells (**bottom left**). MTT assay to evaluate effects of a range of memantine concentrations on viability of clones of interest (**top right**). Effect of memantine on liver metastasis of 2 metastatic isolated clones (**bottom right**).

## Metastasis Gene Signature May Inform Clinical Outcome




(**Left**) The 100 most enriched and 100 most downregulated genes from transcriptomic analysis in DE analysis in the three pro-met subclones versus the three non-met subclones were used to derive a pro-met signature. scRNAseq analysis of parental patient-derived xenograft cell line (n = 3,397) was used to identify clones with metastatic potential. (**Right**) Kaplan-Meier survival analysis of patients in the TCGA pancreatic cancer cohort separated by expression or not of the pro-met transcriptomic signature.

## Conclusions

- Expanding primary tumors are extremely dynamic and undergo a continuous reshuffling of their clonal architecture, even in the absence of any external perturbations, a behavior we named alternating clonal dominance
- Clonal dominance and cell intrinsic long-term replicative potential in the primary tumor are key factors that dictate clonal dissemination to secondary organs.
- Functional upregulation of glutamatergic neural/synaptic signaling in metastatic clones drives invasion and dissemination and represents an actionable therapeutic target to suppress tumor metastasis.
- A transcriptomic signature to detect primary tumor cells with the ability to sustain distant metastases may predict patient survival.