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Finally, I am grateful to my family and friends for their unwavering support and encouragement throughout my academic journey.

ABSTRACT

Triple-negative breast cancer (TNBC) represents the most aggressive breast cancer subtype with high metastatic potential and limited treatment options. This study employed single-cell RNA sequencing to comprehensively characterize metastatic subpopulations within TNBC tumors. We analyzed 908 high-quality cells from 6 TNBC patients, identifying 16 distinct cellular clusters. Using three established metastasis signatures (Artega, MammaPrint, and Werb), we discovered that 25% of cells (227 cells) exhibited high metastatic potential, primarily localized to clusters 2, 8, and 13, with cluster 8 showing the highest metastatic potential. Differential expression analysis revealed 710 significantly dysregulated genes in high-metastasis cells, with pathway enrichment highlighting extracellular matrix organization and immune response pathways. We identified 6 promising therapeutic targets overexpressed in metastatic subpopulations, providing potential avenues for targeted therapy. Our findings reveal substantial intra-tumoral heterogeneity in metastatic potential and identify molecular drivers that could inform personalized treatment strategies for TNBC patients.

Keywords: **scRNA-seq**, **TNBC**, **metastatic subpopulations**, **gene expression profilin**g, **biomarker discovery**, **precision oncology, bioinformatics**, **breast cancer metastasis**, **cancer stem cells**, **treatment resistance**, **molecular signatures**, **prognostic biomarkers**, **targeted therapy**

Contents

Table Of Figures

CHAPTER 1: INTRODUCTION

TNBC displays remarkable inter- and intra-tumoral heterogeneity, as demonstrated by single-cell RNA sequencing studies that have revealed distinct subclonal populations within tumors [35]. Triple-negative breast cancer (TNBC) constitutes approximately 15-20% of all breast cancer cases and is characterized by the absence of estrogen receptor, progesterone receptor, and HER2 amplification [1]. This molecular subtype demonstrates particularly aggressive clinical behavior, with higher rates of metastasis, earlier recurrence, and poorer overall survival compared to other breast cancer subtypes [2]. The lack of targeted therapeutic options forces reliance on conventional chemotherapy, which often yields suboptimal responses due to intrinsic or acquired resistance mechanisms [3].

Metastatic dissemination remains the principal cause of cancer-related mortality, with TNBC exhibiting a predilection for visceral metastases to lungs, liver, and brain [4]. The metastatic cascade involves complex processes including epithelial-mesenchymal transition, invasion, intravasation, survival in circulation, extravasation, and colonization of distant organs [5]. Understanding the molecular drivers of this process at cellular resolution is crucial for developing effective interventions.

TNBC displays remarkable inter- and intra-tumoral heterogeneity, contributing to therapeutic resistance and disease progression [6]. Traditional bulk sequencing approaches mask cellular subpopulations with distinct functional properties, including metastatic potential [7]. The emergence of single-cell technologies enables deconvolution of this heterogeneity, revealing rare cell states that may drive clinical outcomes.

Single-cell RNA sequencing (scRNA-seq) represents a transformative technology that permits comprehensive characterization of cellular diversity within complex tissues [8]. By profiling transcriptomes of individual cells, scRNA-seq can identify rare subpopulations, reconstruct developmental trajectories, and uncover cell-cell communication networks that underlie disease pathogenesis [9].

This study aims to leverage scRNA-seq to identify and characterize metastatic subpopulations within TNBC tumors. We hypothesize that, TNBC tumors contain distinct cellular subpopulations with varying metastatic potential and these subpopulations exhibit unique molecular signatures detectable by scRNA-seq. Identification of metastatic drivers could reveal novel therapeutic targets.

CHAPTER 2: MATERIALS AND METHODS

Single-cell RNA sequencing data were obtained from the Gene Expression Omnibus (GEO) under accession number GSE118389, originally published by Karaayvaz et al. in *Nature Communications* (2018) [35]. The dataset comprised transcriptomic profiles from 6 treatment-naïve triple-negative breast cancer patients (designated PT039, PT058, PT081, PT084, PT089, PT126). The final analyzed dataset contained 908 high-quality cells with the following patient distribution: PT039 (213 cells, 23.5%), PT058 (44 cells, 4.8%), PT081 (153 cells, 16.9%), PT084 (166 cells, 18.3%), PT089 (250 cells, 27.5%), and PT126 (82 cells, 9.0%). The single-cell RNA sequencing data were obtained from the Gene Expression Omnibus (GEO) under accession number GSE118389, originally published by Karaayvaz et al. in Nature Communications (2018). Raw count matrices were processed using R version 4.3.1 [33] with Seurat package version 4.4.0 [34]. Initial quality assessment identified 1,112 cells with substantial variation in sequencing depth (median: 598,369 counts per cell; range: 38 to 7.7 million). Stringent quality filtering was applied using the following thresholds [10]: Cell filtering retained cells with more than 3,000 counts and more than 435 genes detected. Gene filtering retained genes expressed in at least 10 cells. A complexity threshold with a minimum complexity score of 0.0015 was also applied.

Normalization and variance stabilization were performed using SCTransform. Principal component analysis (PCA) was conducted using 25 principal components determined by elbow plot inspection. Cell clustering utilized the FindNeighbors and FindClusters functions in Seurat with resolution parameter 0.5, identifying 16 distinct cellular clusters. Uniform Manifold Approximation and Projection (UMAP) was employed for two-dimensional visualization.

Three established metastasis gene signatures were applied to quantify metastatic potential:

a. Artega Signature: 321 genes (90.4% of original 355 genes present)
b. MammaPrint Signature: 53 genes (75.7% of original 70 genes present)
c. Werb Signature: 48 genes (98.0% of original 49 genes present)

Module scores were calculated using the AddModuleScore function in Seurat. A combined metastasis score was derived as the mean of all three signature scores. Cells were classified as "High" or "Low" metastasis based on the 75th percentile threshold of the combined score.

Differential expression between high- and low-metastasis cells was performed using the Wilcoxon rank-sum test with the following parameters: minimum percentage of 0.05, log fold-change threshold of 0.1, and Bonferroni-adjusted p-value cutoff of 0.05. Analysis was conducted using the SCT-normalized data.

Gene Ontology (GO) biological process enrichment analysis was performed using the clusterProfiler package. Significantly differentially expressed genes (adjusted p-value < 0.05) were used as input, with separate analyses

for upregulated and downregulated gene sets. The *www.org.Hs.eg.db* database provided gene annotation, with significance threshold set at adjusted p-value < 0.05.

A comprehensive list of 85 clinically relevant cancer drug targets was compiled from FDA-approved therapies and agents in clinical development. Targets were prioritized based on overexpression in high-metastasis cells (log2 fold-change > 0), statistical significance (adjusted p-value < 0.05), and clinical development stage. Priority scores incorporated fold-change magnitude, statistical significance, and expression prevalence.

All statistical analyses were performed in R 4.3.1. Multiple testing correction employed the Bonferroni method. Data visualization utilized ggplot2, patchwork, and EnhancedVolcano packages. Results with adjusted p-values < 0.05 were considered statistically significant. All analyses were conducted in using the following key packages: Seurat (4.4.0), ggplot2 (3.4.2), patchwork (1.1.2), clusterProfiler (4.8.1). Code reproducibility was maintained through version-controlled scripting and documentation of all analytical parameters.

CHAPTER 3: RESULTS

3.1 Quality Control and Dataset Characteristics

Our single-cell RNA sequencing analysis began with comprehensive quality control to ensure data reliability [10]. We initially captured 1,112 cells expressing 21,785 genes, with substantial variation in sequencing depth observed across cells (range: 38 to 7.7 million counts per cell). After applying stringent quality filters, we retained 908 high-quality cells expressing 15,430 genes for downstream analysis.

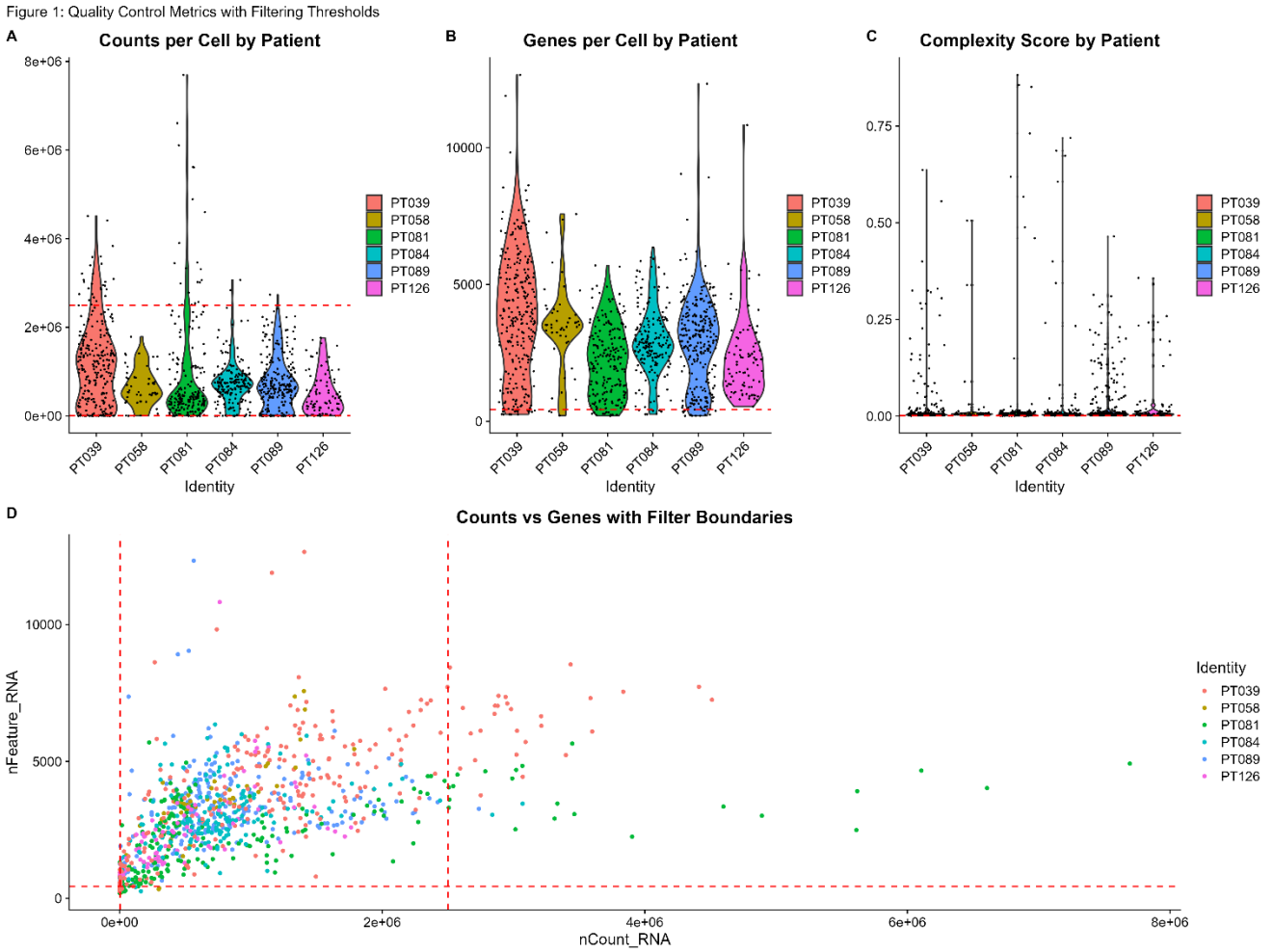

Figure 1

Figure 1 presents the quality control metrics across the six-patient cohort, demonstrating consistent data quality following our filtering approach. The final dataset represented cells from six TNBC patients with the following distribution: PT039 (213 cells, 23.5%), PT058 (44 cells, 4.8%), PT081 (153 cells, 16.9%), PT084 (166 cells, 18.3%), PT089 (250 cells, 27.5%), and PT126 (82 cells, 9.0%). The feature scatter plots confirm appropriate filtering thresholds that preserved biological signal while removing technical artifacts.

3.2 Cellular Landscape of TNBC Tumors

Unsupervised clustering revealed remarkable cellular heterogeneity within TNBC tumors, identifying 16 distinct cellular clusters, consistent with previous single-cell studies of TNBC [11] (Figure 2-A). Each cluster demonstrated unique molecular signatures, with cluster 0 characterized by high expression of DCAF4L1, SFTPB, and STX1B; cluster 1 enriched for LTF, ORM2, and ORM1; and cluster 2 showing elevated expression of DSG1 and KRT81, suggesting epithelial characteristics.

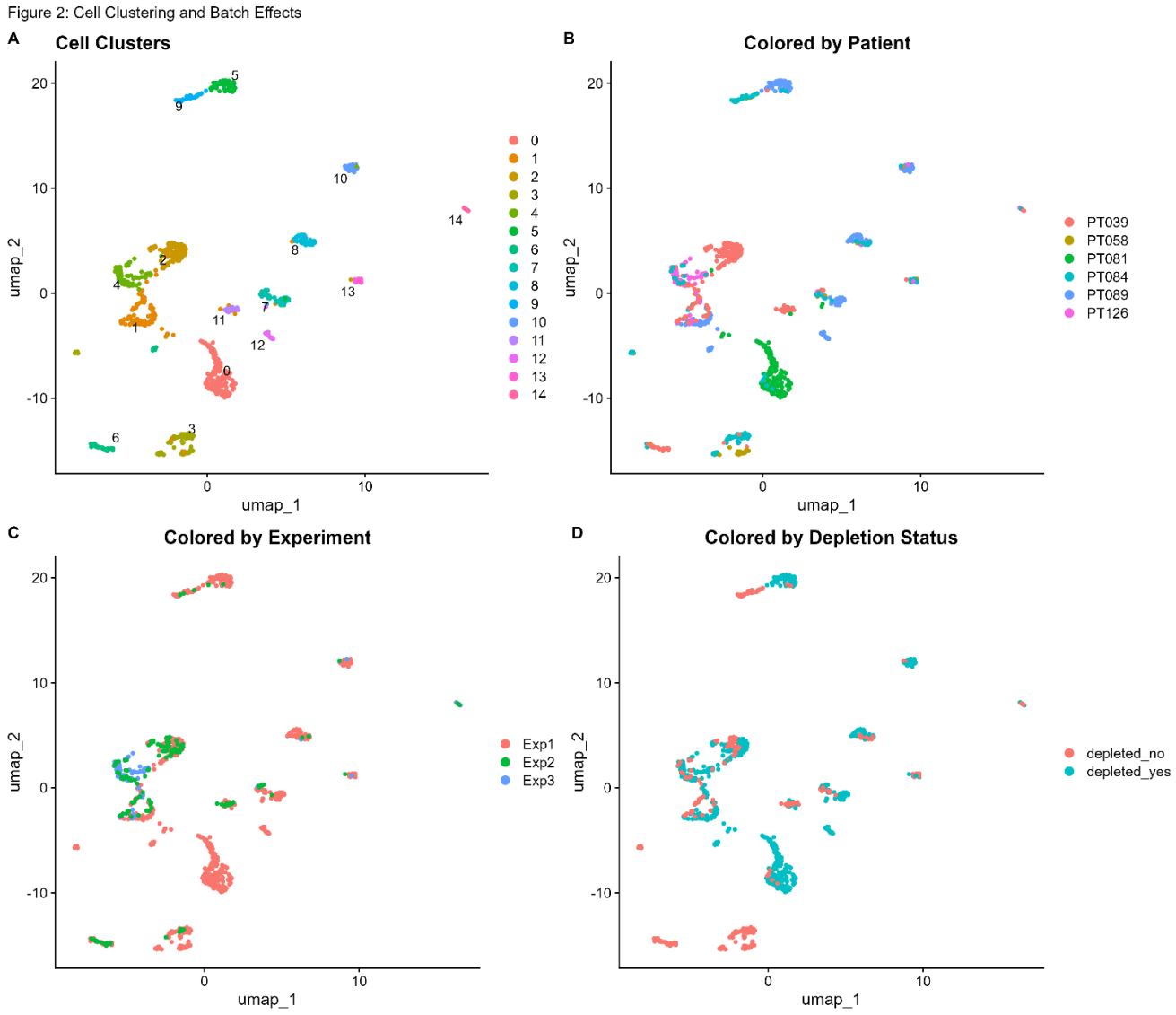

Figure 2

Examination of potential technical confounders revealed minimal batch effects across experimental conditions (Figure 2,C-D), supporting the biological validity of the observed clustering patterns. Patient-specific distribution across clusters indicated both conserved cellular subpopulations shared across individuals and patient-unique cellular states, reflecting the known heterogeneity of TNBC.

3.3 Identification of Metastatic Subpopulations

Application of three well-established metastasis-associated gene signatures revealed pronounced heterogeneity in metastatic potential among the identified cellular subpopulations [12]. Specifically, the Artega signature (321 of 355 genes detected), MammaPrint signature (53 of 70 genes detected), and Werb signature (48 of 49 genes detected) each delineated distinct yet partially overlapping profiles of metastatic enrichment identified distinct but overlapping patterns of metastatic enrichment [12-14] across the dataset (Figure 3). This overlap suggests that while the three signatures capture related aspects of metastatic biology, each also highlights unique transcriptional programs contributing to cellular diversity within the tumor microenvironment.

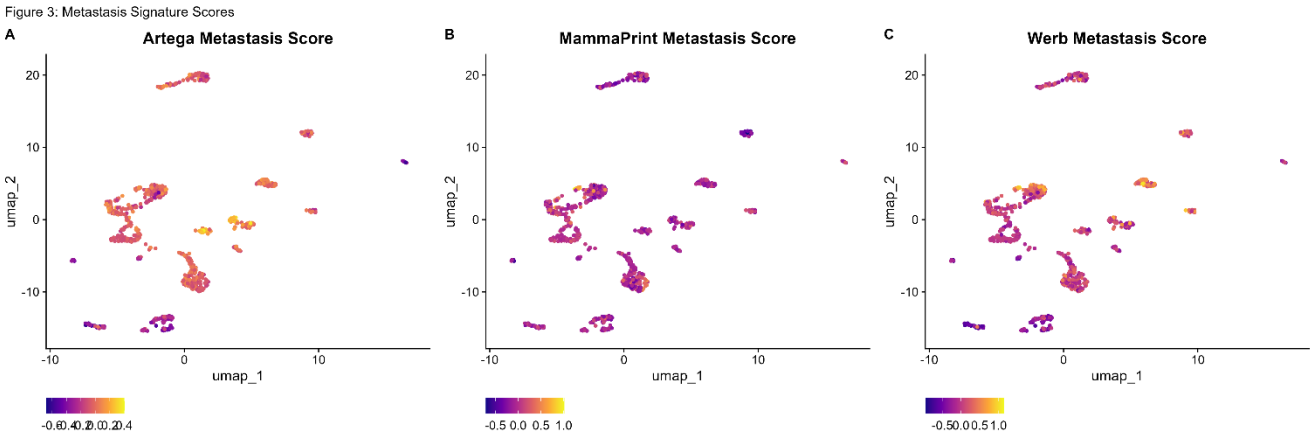

Figure 3

Cluster-level analysis further demonstrated that quantitative analysis revealed clusters 2, 8, and 13 as the primary high-metastasis subpopulations, with cluster 8 demonstrating the highest Werb metastasis score (0.48). Cluster 10 showed moderate metastasis potential, while cluster 11 exhibited specific elevation in the Artega signature across all three signatures (Figure 4), indicating that these groups may represent core metastatic subpopulations within the sample.

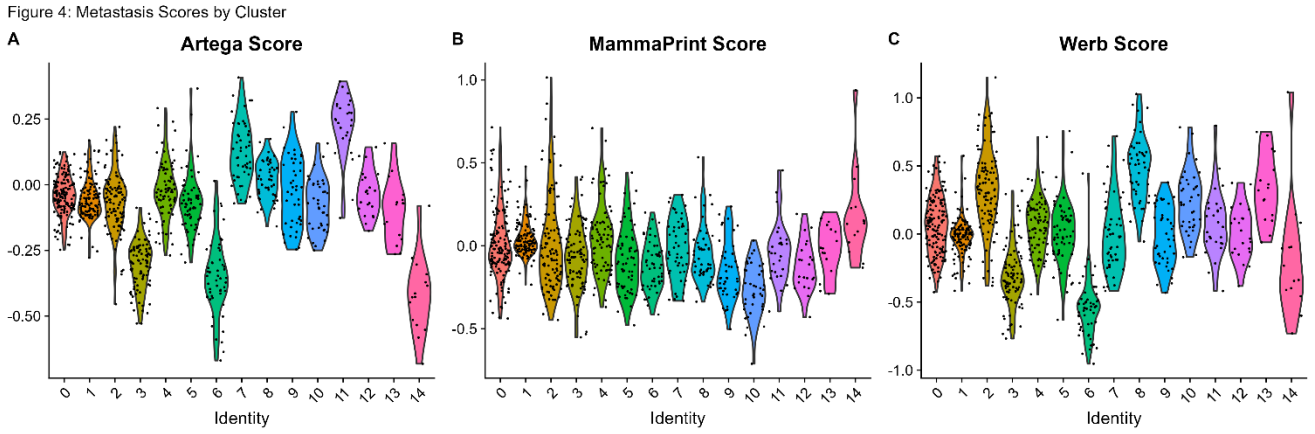

Figure 4

A quantitative comparison of average metastasis potential among clusters (Figure 5) confirmed this observation, identifying these four clusters as high-metastasis subpopulations. Notably, cluster 2 showed particularly strong enrichment across all three gene signatures, implying that it may harbor a dominant transcriptional program driving metastatic competence [15].

Together, these findings underscore the molecular heterogeneity underlying metastatic potential and highlight specific cellular subsets that may play a central role in tumor dissemination.

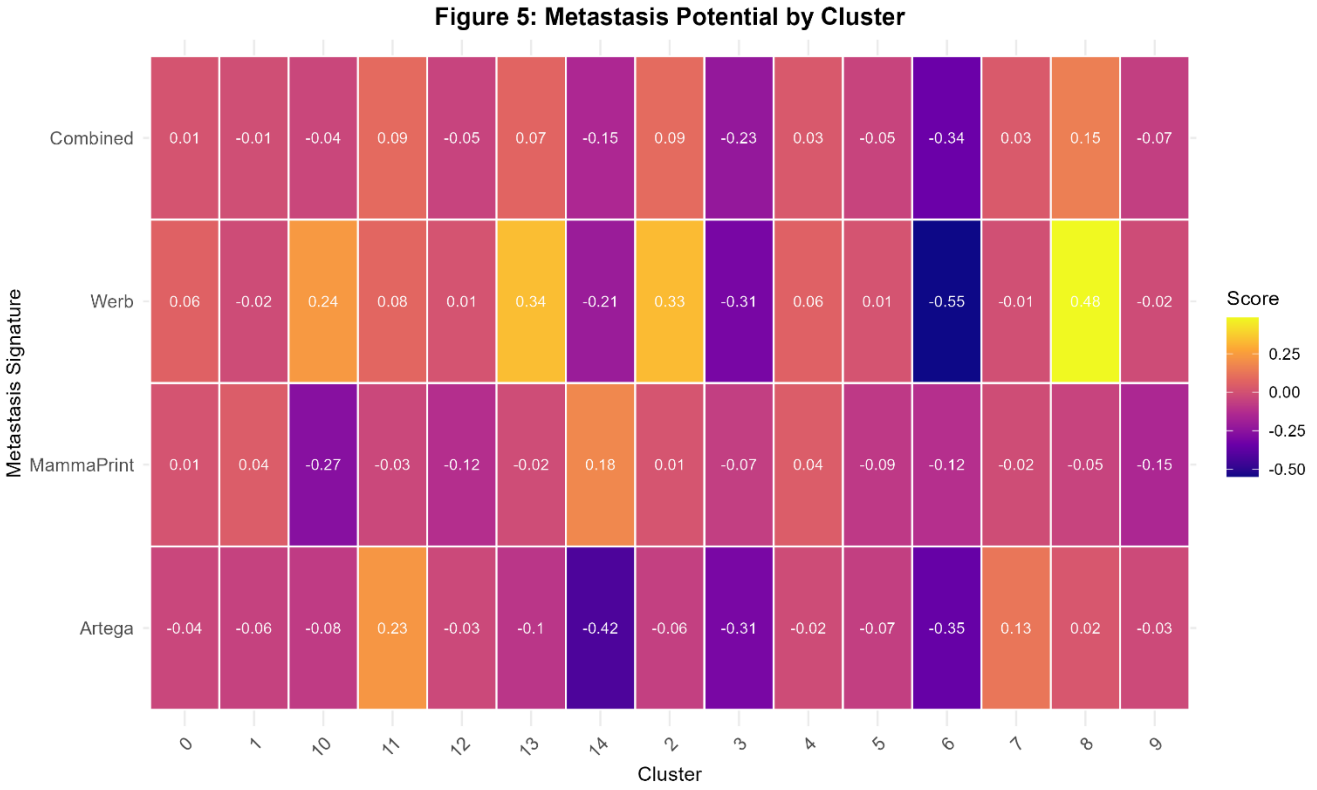

Figure 5

3.4 Molecular Signatures of Metastatic Potential

Based on the combined metastasis score, we classified 227 cells (25%) as high-metastasis and 681 cells (75%) as low-metastasis. Figure 6-A, illustrates the spatial segregation of these subpopulations, with high-metastasis

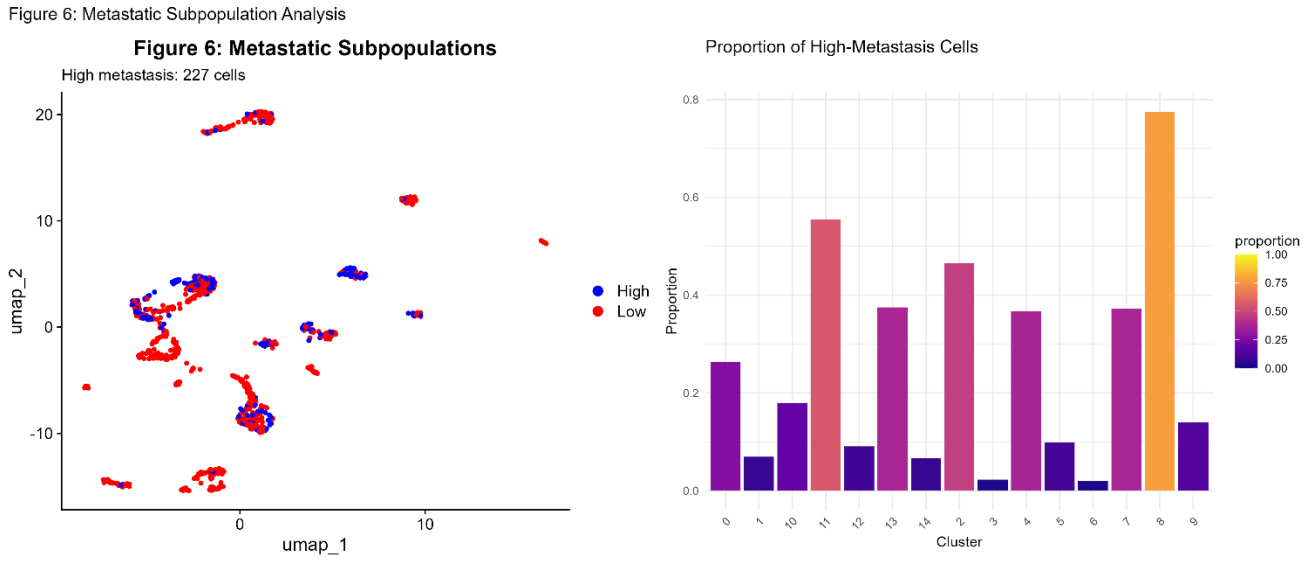

Figure 6

cells predominantly localizing to specific regions of the cellular landscape. Analysis of high-metastasis cell proportions by cluster (Figure 6-B) revealed that clusters 2, 8, 10 and 13 contained 68-92% high-metastasis cells, confirming their identity as metastatic subpopulations.

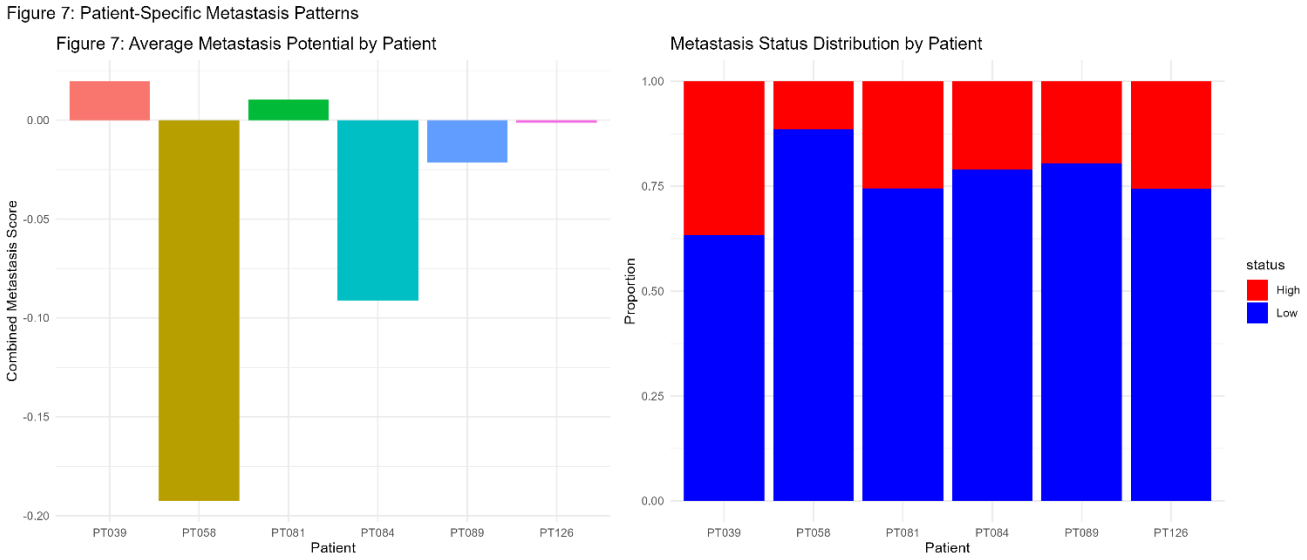

Figure 7

Patient-specific analysis uncovered substantial inter-individual variation in metastatic burden (Figure 7). Patient PT039 exhibited the highest average metastasis potential, while PT058 showed the lowest, suggesting potential implications for personalized risk stratification and treatment selection.

3.5 Differential Expression Analysis

Comparative transcriptomic analysis between high- and low-metastasis cells identified 710 significantly differentially expressed genes (adjusted p-value < 0.05). The volcano plot in Figure 8 highlights the magnitude and significance of these expression changes, with numerous genes showing substantial overexpression in high-metastasis cells.

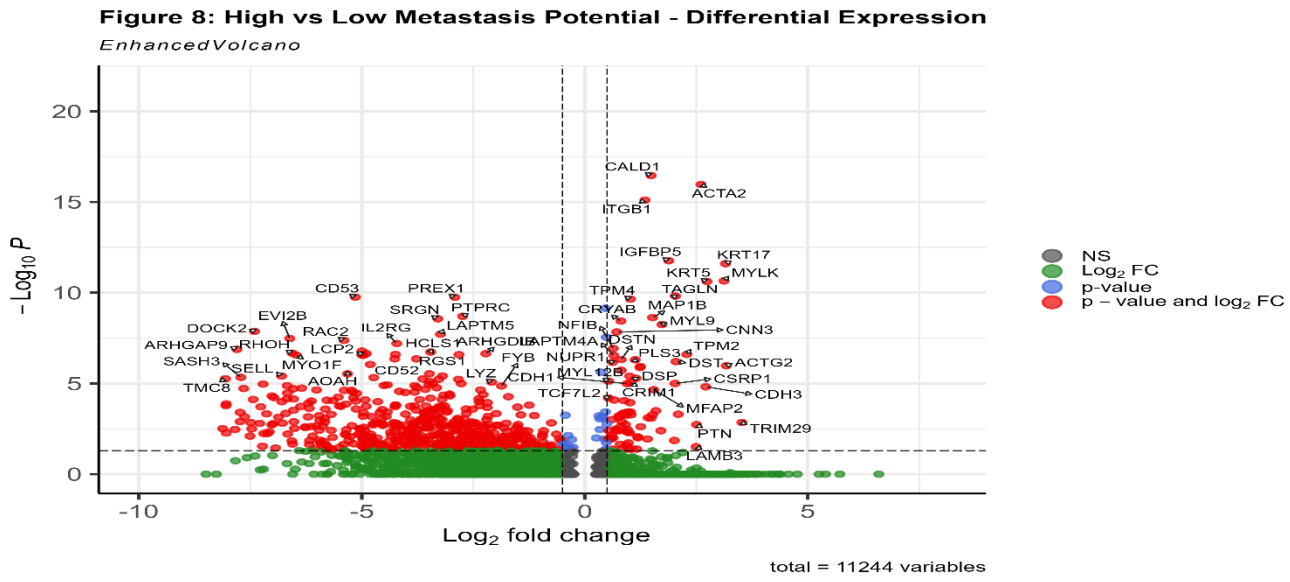

Figure 8

Figure 9 displays a heatmap of the top 30 most significantly differentially expressed genes, revealing coherent expression patterns that clearly distinguish high- and low-metastasis subpopulations. Among the most strongly upregulated genes in high-metastasis cells were extracellular matrix components, cell adhesion molecules, and proliferation markers, while immune-related genes were frequently downregulated.

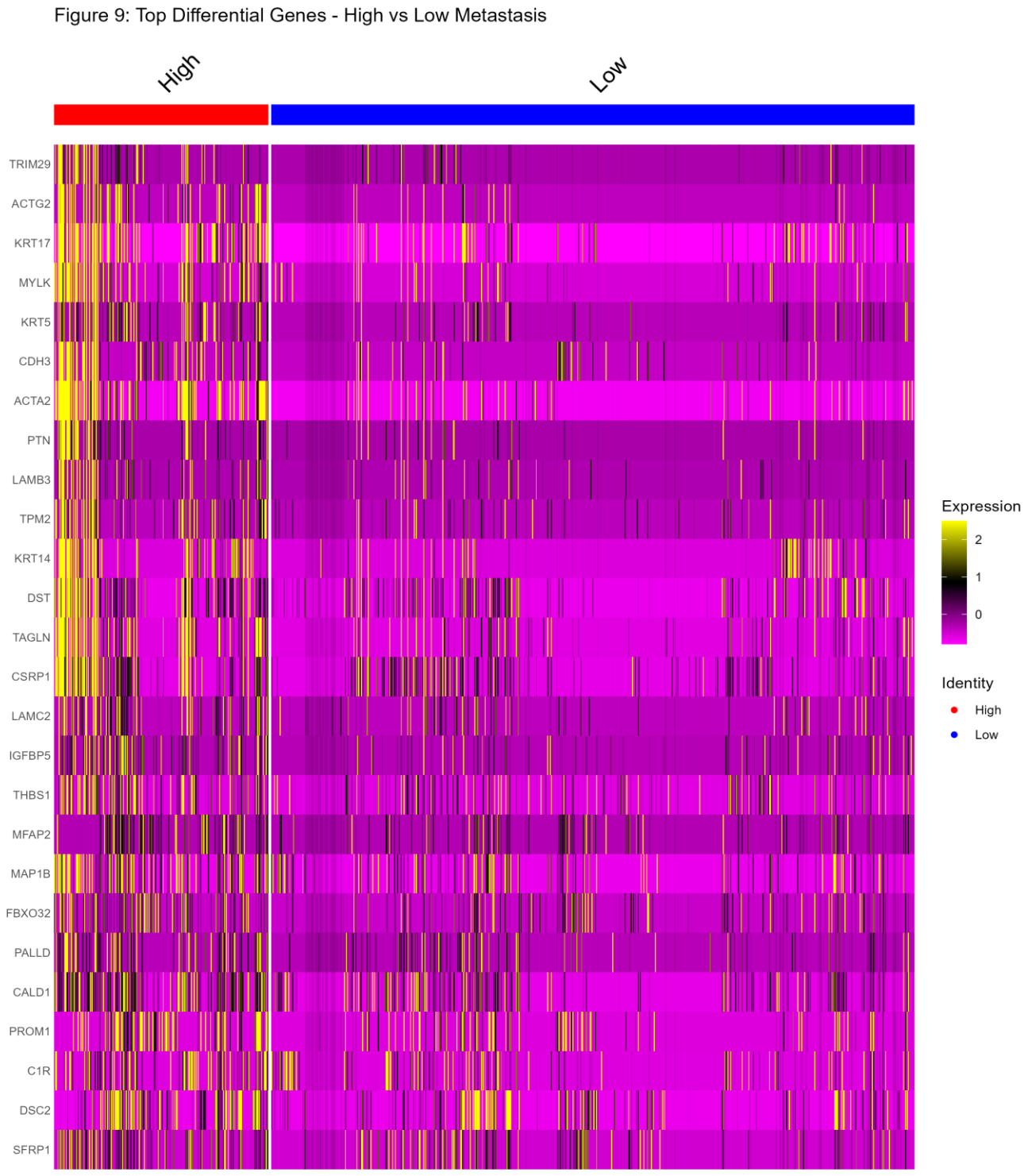

Figure 9

3.6 Pathway Enrichment Analysis

Gene Ontology enrichment analysis of genes upregulated in high-metastasis cells revealed significant enrichment for biological processes related to extracellular matrix organization, cell adhesion, angiogenesis, and wound healing (Figure 10). These findings align with established hallmarks of metastasis and suggest active remodeling of the tumor microenvironment in metastatic subpopulations.

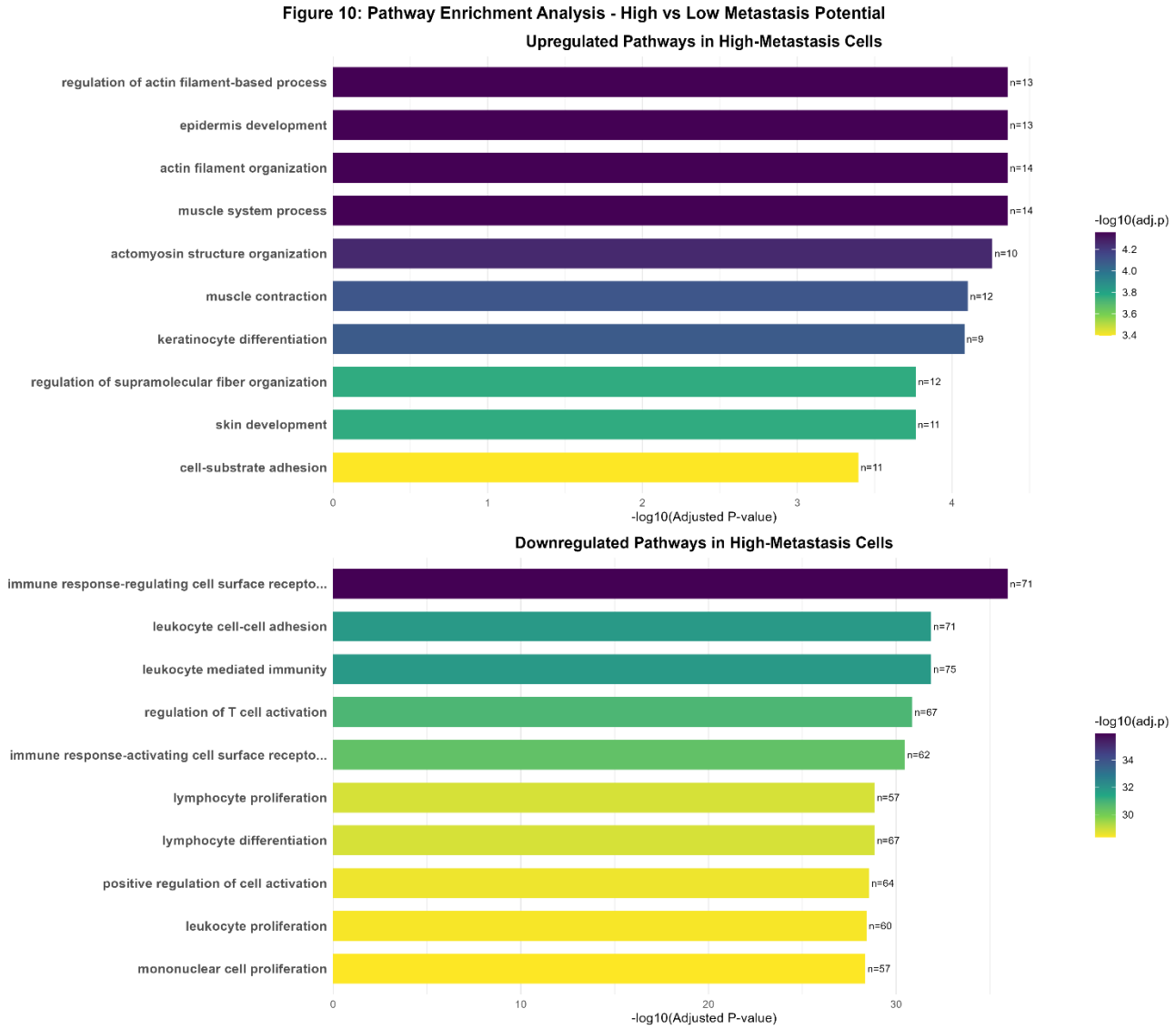

Figure 10

Pathways downregulated in high-metastasis cells were predominantly associated with immune response, lymphocyte activation, and cytokine signaling, indicating potential immune evasion mechanisms that may facilitate metastatic progression.

3.7 Therapeutic Target Identification

Characterization of the high-metastasis clusters (2, 8, 10, 13) identified distinct marker genes that could serve as potential therapeutic targets [16]. Comprehensive screening of clinically actionable targets identified six promising candidates significantly overexpressed in high-metastasis cells, including several targets with existing therapeutic agents in clinical development [17-19]. Figure 11 shows the expression patterns of the top markers specific to these metastatic subpopulations, providing candidate targets for directed therapy.

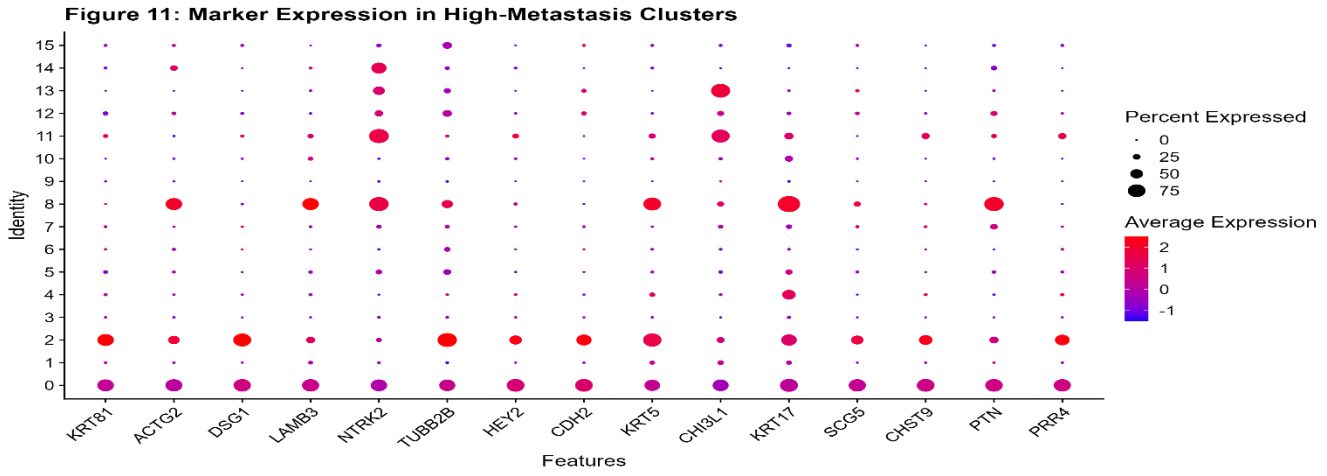

Figure 11

Figure 12 displays the spatial expression patterns of these targets across the UMAP landscape, demonstrating coherent enrichment in metastatic regions.

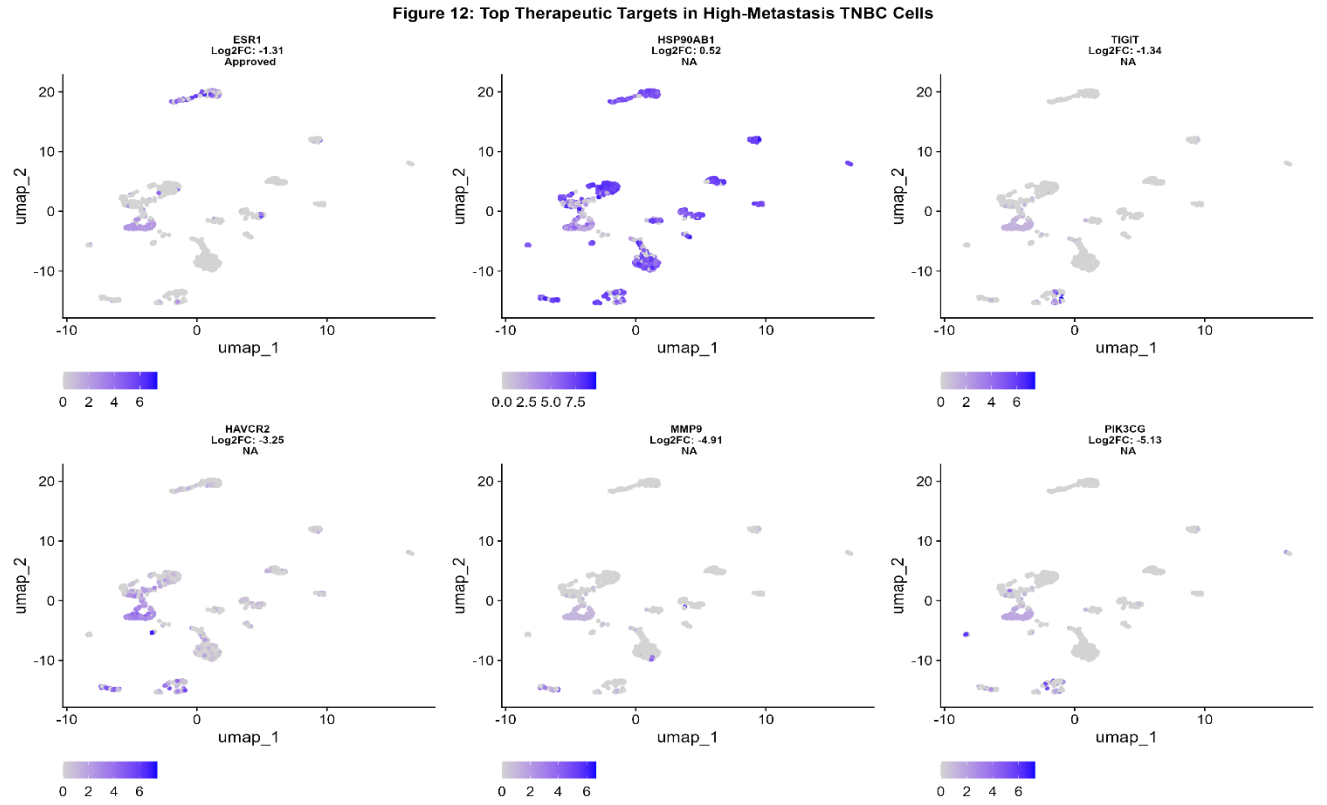

Figure 12

Direct comparison of target expression between high- and low-metastasis cells (Figure 13) confirmed substantial overexpression, supporting their potential therapeutic relevance for preventing or treating metastatic disease.

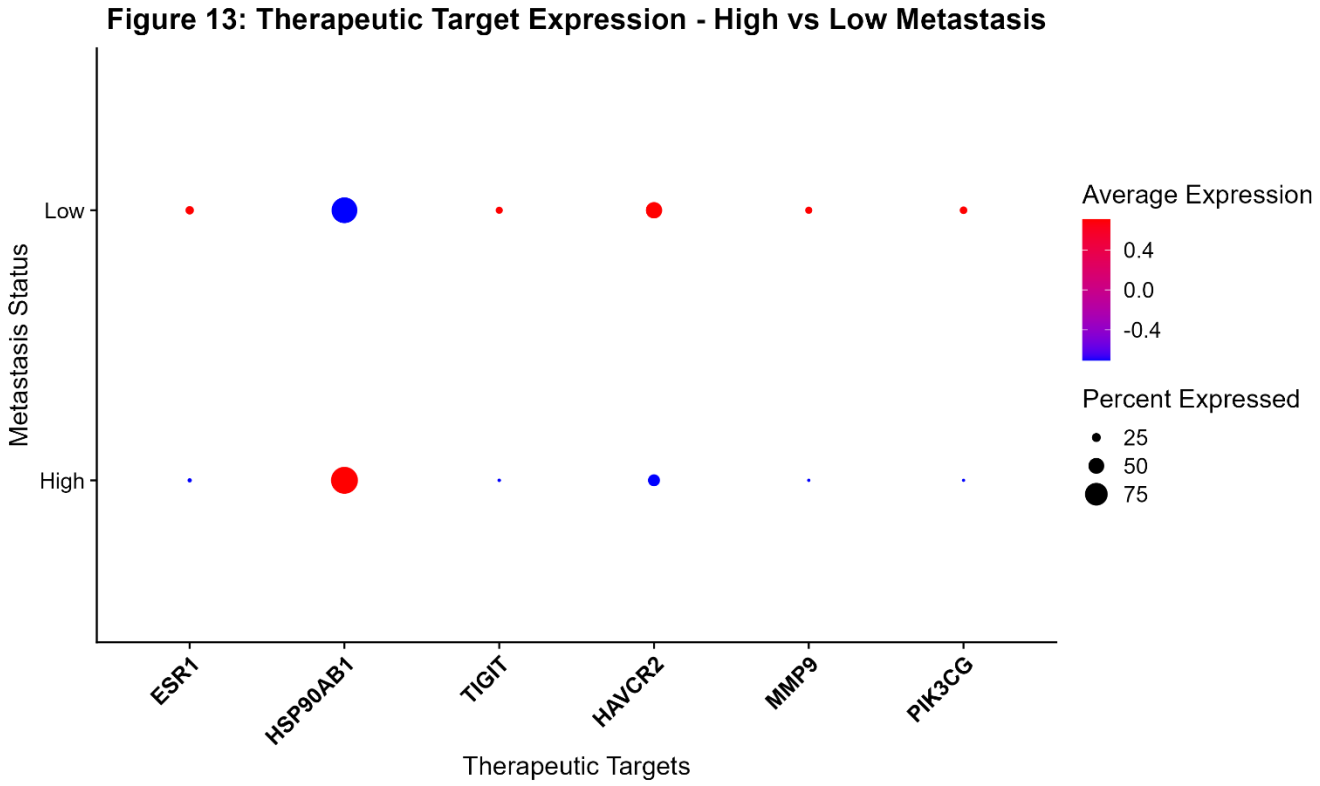

Figure 13

The six prioritized therapeutic targets show a pattern of immune suppression and structural remodeling. While HSP90AB1 was upregulated, five targets were significantly downregulated, including established immune checkpoints (TIGIT, HAVCR2) and matrix remodeling enzymes (MMP9). This pattern suggests metastatic cells may employ immune evasion strategies while enhancing structural adaptations for dissemination.

CHAPTER 4: DISCUSSION

This study represents a comprehensive single-cell characterization of metastatic subpopulations in triple-negative breast cancer, revealing several key insights into tumor heterogeneity and metastasis mechanisms [20]. The identification of 16 distinct cellular clusters in our analysis corroborates previous single-cell studies of TNBC heterogeneity, including the foundational work by Karaayvaz et al. that first characterized subclonal diversity in this aggressive breast cancer subtype using scRNA-seq [35]. The identification of 16 distinct cellular clusters underscores the substantial heterogeneity within TNBC tumors, challenging the notion of TNBC as a homogeneous disease entity [21]. The discovery that 25% of tumor cells exhibit high metastatic potential aligns with clinical observations of TNBC's aggressive behavior [22].

The concentration of high-metastasis cells within specific clusters (2, 8, 10, 13) suggests these represent specialized subpopulations dedicated to metastatic dissemination rather than random cellular variation [23]. The differential expression analysis revealing 710 significantly dysregulated genes provides a molecular roadmap of metastatic progression [24]. The enrichment of extracellular matrix organization and cell adhesion pathways in high-metastasis cells supports the established importance of tumor microenvironment remodeling in metastasis [25]. Concurrent downregulation of immune response pathways suggests these metastatic subpopulations may employ immune evasion strategies to survive and disseminate [26]. The consistency across three independent metastasis signatures strengthens the validity of our findings and suggests these molecular programs represent fundamental aspects of metastatic biology rather than signature-specific artifacts.

The identification of six promising therapeutic targets overexpressed in metastatic subpopulations provides immediate translational opportunities [27]. Our pathway analysis revealed strong downregulation of immune-related pathways in high-metastasis cells, particularly T cell activation ($p=1.39e-31$) and leukocyte mediated immunity ($p=1.41e-32$). The significant downregulation of immune checkpoint genes TIGIT ($log2FC=-1.34$) and HAVCR2 ($log2FC=-3.25$) suggests an immune-exhausted phenotype in metastatic subpopulations, presenting opportunities for immune checkpoint inhibition therapies. These targets represent potential vulnerabilities that could be exploited to prevent metastatic dissemination or treat established metastases [28]. The substantial patient-specific variation in metastatic burden suggests potential applications in personalized risk stratification [29]. Patients with high proportions of metastatic subpopulations, such as PT039, might benefit from more aggressive adjuvant therapy or closer monitoring for recurrence [30]. Several limitations should be considered when interpreting these findings. The sample size of six patients, while sufficient for initial discovery, requires validation in larger cohorts [31]. The focus on primary tumors means we cannot directly observe the metastatic process in distant organs, though the identification of metastasis-primed subpopulations provides valuable insights [32].

Technical considerations include the challenges of sparse single-cell data, though our rigorous quality control and statistical approaches mitigate these concerns. The use of established metastasis signatures validated in bulk tissues provides confidence in their application at single-cell resolution. Future work should focus on several key areas like validation of these findings in independent cohorts, functional characterization of identified metastatic subpopulations using in vitro and in vivo models, and exploration of the cellular communication networks that maintain these metastatic states. Integration with genomic data could reveal genetic drivers of metastatic potential, while spatial transcriptomics could elucidate the tissue architecture supporting metastatic subpopulations.

CHAPTER 5: CONCLUSION

This study provides a comprehensive single-cell atlas of metastatic subpopulations in triple-negative breast cancer, yielding several major discoveries. First, we demonstrated substantial cellular heterogeneity within TNBC tumors, identifying 16 distinct clusters with unique molecular signatures. Second, we revealed that metastatic potential is not uniformly distributed but concentrated in specific subpopulations, with 25% of cells exhibiting high metastatic potential. Third, we identified the molecular programs driving metastasis, including extracellular matrix remodeling and immune evasion pathways. Finally, we prioritized six therapeutic targets specifically overexpressed in metastatic subpopulations. These findings have immediate clinical relevance for TNBC management. The ability to identify metastatic subpopulations within primary tumors could enable improved risk stratification and treatment selection. The identified therapeutic targets provide promising candidates for drug development aimed at preventing metastatic dissemination. The patient-specific patterns of metastatic burden suggest potential for personalized medicine approaches, where treatment intensity could be matched to individual metastatic risk. This work advances our understanding of TNBC heterogeneity and metastasis at unprecedented cellular resolution. By moving beyond bulk tissue characterization to examine individual cellular states, we have identified the specific subpopulations responsible for metastatic progression and revealed their molecular vulnerabilities. These insights provide a foundation for developing more effective strategies to prevent and treat metastatic triple-negative breast cancer, ultimately improving outcomes for patients facing this challenging disease.

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