

Comparative Proteomic Profiling of Microdissected Spindle and Epithelioid Uveal Melanoma Subtypes

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PURPOSE. Uveal melanoma (UM), the most prevalent primary intraocular cancer in adults, is defined by salient histopathological diversity. Spindle and epithelioid cells constitute its two dominant pathological lineages, yet the latter foreshadows aggressive behavior and shortened survival. This study aimed to explore the molecular and metabolic underpinnings of UM pathology using regionally resolved proteomics.

METHODS. Formalin-fixed, paraffin-embedded tumors from four patients—two spindle cell and two epithelioid—were histologically stratified via hematoxylin and eosin staining and subsequently interrogated by regionally resolved proteomics across eight precisely mapped regions. Immunofluorescence corroborated the shifts in melanocyte lineage markers. To translate these tissue-level signatures into functional biology, spindle-like 92.1 and epithelioid Mel290 cell lines were subjected to quantitative RT-PCR and Seahorse metabolic profiling, quantifying oxidative-phosphorylation (OXPHOS) gene expression and mitochondrial respiration.

RESULTS. Proteomic profiling of microdissected tissue uncovered a selective collapse of the melanocytic differentiation program within epithelioid foci. Endothelin receptor type B, the receptor required for melanocyte stem cell fate, and the lineage-defining calcium-binding protein S100B were both sharply repressed in epithelioid samples, with S100B registering the single largest drop across the entire dataset. Immunofluorescence corroborated this signature, revealing concomitant loss of microphthalmia-associated transcription factor and premelanosome protein, further attesting to the dedifferentiated state of epithelioid cells. Simultaneously, the same regions exhibited a striking surge in OXPHOS machinery. Concordantly, Mel290 epithelioid cells displayed elevated transcription of OXPHOS genes and a markedly higher basal and maximal oxygen consumption rate compared with the spindle-like 92.1 line, aligning tissue-level proteomic shifts with cell-autonomous metabolic rewiring.

CONCLUSIONS. Our findings reveal a distinct proteomic signature in epithelioid UM characterized by dedifferentiation and enhanced mitochondrial respiration. This study provides the first regionally resolved proteomic landscape of UM pathology and suggests that the epithelioid transformation may reflect a shift toward a dedifferentiated, metabolically active tumor state.

Keywords: uveal melanoma, histopathology, proteomic profiling, dedifferentiation, metabolic switch

Uveal melanoma (UM) is the most common primary intraocular malignancy in adults, arising from melanocytes in the uveal tract. Although it is a rare cancer with an incidence of approximately five to seven cases per million per year in Western countries, its clinical significance lies in its high potential for metastatic spread, particularly to the liver, which accounts for the majority of UM-related mortality.^{1,2} Despite effective local control achieved through treatments such as plaque brachytherapy, proton beam radiotherapy, and enucleation, up to 50% of patients eventually develop metastases, leading to poor survival outcomes.^{3,4}

Histopathological classification of UM offers valuable prognostic information. Spindle-type UM, defined by an elongated, fusiform cell morphology, are generally associated with a more favorable prognosis. In contrast, epithelioid UM cells, characterized by large, round nuclei and prominent nucleoli, are linked to greater aggressiveness and metastatic risk. Mixed tumors contain both cell types and display intermediate outcomes.^{4–6} However, the molecular mechanisms underlying the emergence and behavior of these histopathological subtypes have remained elusive.

The melanocytic lineage is governed by a tightly regulated differentiation program driven by the microphthalmia-associated transcription factor (MITF), often termed the master regulator of melanocyte identity.⁷ MITF orchestrates the expression of pigment-producing enzymes and structural proteins essential for melanosome biogenesis, including tyrosinase, tyrosinase-related protein 1, dopachrome tautomerase, and premelanosome protein (PMEL).⁸ Beyond pigmentation machinery, lineage specification relies on signaling receptors such as endothelin receptor type B (EDNRB) and calcium-binding proteins like S100B, which act to maintain melanocyte survival and stemness.^{9,10}

Concurrently, metabolic reprogramming has emerged as a critical determinant of tumor progression. Accumulating evidence suggests that UM tumors, particularly those with high-risk molecular features, display significant metabolic diversity. Recent bioenergetic profiling has indicated that highly metastatic monosomy 3 UM subsets exhibit a high mitochondrial reserve capacity and increased expression of electron transport chain components, such as succinate dehydrogenase A.¹¹ Furthermore, BAP1-mutant UM has been shown to exhibit intrinsic metabolic heterogeneity, resulting in distinct subgroups with specific sensitivities to metabolic inhibitors.¹² Elucidating these metabolic profiles is crucial for translating biological insights into targeted interventions that leverage the unique metabolic dependencies of UM.¹³

Recent advances in regionally resolved transcriptomics and proteomics have enabled a deeper understanding of tissue heterogeneity by preserving regional context while quantifying molecular features. Laser capture microdissection (LCM)-based proteomics, in particular, offers high-resolution mapping of protein expression patterns directly within histological sections, thus providing unprecedented insight into key mechanisms driving tumor development and progression.^{14–17} In the context of UM, previous proteomic studies have offered valuable molecular profiling at the bulk tumor level,^{18,19} but have largely overlooked histopathological heterogeneity, particularly the distinctions between spindle and epithelioid cell types.

In this study, we performed proteomic profiling of microdissected UM samples enriched for distinct histopatho-

logical cell types to characterize their molecular signatures. We discovered that epithelioid tumors exhibit significant downregulation of melanocyte differentiation markers, including EDNRB and S100B, alongside increased oxidative-phosphorylation (OXPHOS) activity, suggesting a shift toward a dedifferentiated and metabolically reprogrammed state. These findings were validated by immunofluorescence and further supported by *in vitro* assays using representative UM cell lines. Conclusively, this study presents the first regionally resolved proteomic landscape of histopathological heterogeneity in UM, offering mechanistic insights into the molecular divergence between spindle and epithelioid tumor cells and illuminating potential pathways driving disease progression.

METHODS

Sample Collection and Ethical Approval

Human tumor specimens were obtained from UM patients who underwent enucleation between August 2022 and August 2024. Histopathological classification of cell types was confirmed by the Department of Pathology at Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine. Clinicopathological data were collected through a comprehensive review of patients' medical records and imaging results. The data were analyzed in accordance with the principles of the Declaration of Helsinki. The study protocol was approved by the Institutional Review Board of the Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (SH9H-2019-T185-2). Participants gave informed consent to participate in the study before taking part.

Tissue Processing and LCM

Formalin-fixed, paraffin-embedded (FFPE) tissue sections were cut at 8 μ m to ensure sufficient protein yield for downstream mass spectrometry while maintaining histological resolution for cell-type identification. After air-drying and incubation at 65°C for 1 hour, sections were deparaffinized in xylene and rehydrated through a graded ethanol series (100%, 95%, 85%, 75%, and 50%). Hematoxylin and eosin staining was performed by incubating sections with hematoxylin for 7 minutes at room temperature, rinsing with ultrapure water, applying bluing buffer for 2 minutes, and then staining with eosin for 40 seconds. After a final rinse with ultrapure water, slides were air-dried on a clean, flat surface. Stained slides were scanned at 20 $\times$ magnification using the SLIDEVIEW VS200 digital slide scanner (Olympus, Tokyo, Japan).

LCM was conducted using the PALM laser microdissection system (Zeiss, Jena, Germany). The machine was prewarmed for 15 minutes to stabilize the laser, and parameters were set as follows: zoom, 20 $\times$; cut energy, 39; focus, 62; catapulting energy, 64; focus, 70; cycle number, 1; and cut speed, 100. The regions of interest containing a specific cell type in the section were marked using an LCM marker pen, microdissected with the previously described settings, and collected into microtubes (Zeiss, 415190-9201-000).

Protein Digestion and Mass Spectrometry

Microdissected tissues were resuspended in 5 μ L of lysis buffer, sonicated for 3 minutes using a contactless high-

intensity ultrasonic processor (Scientz, Ningbo, China), and incubated at 95°C for 5 minutes. Protein digestion was carried out overnight at 37°C with 1 μ L of 50 ng trypsin. Peptides were desalted using C18 ZipTips (per the manufacturer's instructions) and dried for subsequent liquid chromatography-tandem mass spectrometry analysis.

Peptide separation was performed using a Vanquish Neo UPLC system (ThermoFisher Scientific, Waltham, MA, USA) at a flow rate of 200 nL/min, following the manufacturer's protocol. The eluted peptides were analyzed on an Orbitrap Astral mass spectrometer equipped with a nano-electrospray ion source (1900 V). Precursor ions were detected in the Orbitrap at a resolution of 240,000 over a scan range of 400 to 800 m/z, and fragment ions were analyzed in the Astral detector. Mass spectrometry acquisition was performed with a first mass of 150.0 m/z at a resolution of 80,000, using high-energy collision dissociation at a normalized collision energy of 25%. The automatic gain control target was set to 800%, with a maximum injection time of 15 ms.

The DIA data were processed using the DIA-NN search engine (v.1.8). Tandem mass spectra were searched against the Homo sapiens proteome database (FASTA file: Homo_sapiens_9606_SP_20241202.fasta, 20,422 entries) concatenated with a reverse decoy database. Trypsin/P was specified as the cleavage enzyme, allowing up to one missing cleavage. Cysteine carbamidomethylation and N-terminal methionine excision were specified as fixed modifications. The false discovery rate was adjusted to less than 1%. Protein quantification was performed using the specific DIA-NN algorithm. Subsequent data normalization was performed using the global normalization setting in DIA-NN, and missing values were excluded from analysis if present in >50% of samples.

Functional Enrichment and Clustering

Fisher's exact test was used to assess the significance of functional enrichment among differentially expressed proteins, using all identified proteins as the background. *P* values were adjusted for multiple comparisons using the Benjamini-Hochberg method. Enriched terms with a fold enrichment of greater than 1.5 and a false discovery rate of less than 0.05 were considered significant. For hierarchical clustering based on functional classifications (e.g., Gene Ontology, Kyoto Encyclopedia of Genes and Genomes), enriched categories and their associated *P* values were compiled. Categories enriched in at least one cluster ($P < 0.05$) were retained, and the resulting *P* value matrix was transformed using $-\log_{10}(P)$. One-way hierarchical clustering was performed using Euclidean distance and average linkage in Genesis. Cluster memberships were visualized as a heatmap using the Heatmap function from the ComplexHeatmap R package.

For pathway-level functional analysis, gene set enrichment analysis was performed using the Kyoto Encyclopedia of Genes and Genomes pathway database. Significance was determined using a gene set permutation test, and *P* values were adjusted using the Benjamini-Hochberg procedure. Pathways with a false discovery rate of less than 0.05 and significant normalized enrichment scores (NES) were considered biologically relevant. Additionally, to visualize pathway activity landscapes across individual samples, single-sample gene set enrichment analysis was performed

using the tool at https://github.com/broadinstitute/ssGS_EA2.0.

Immunofluorescence Staining Assay

Deparaffinized and rehydrated tissue sections were fixed in 4% formaldehyde for 30 minutes, blocked with 5% normal goat serum (Vector Labs, Burlingame, CA, USA) for 1 hour, and permeabilized using 0.5% Triton X-100 for 15 minutes. Samples were then incubated with primary antibodies overnight at 4°C, followed by incubation with corresponding secondary antibodies for 1 hour at room temperature. Nuclei were counterstained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes at room temperature. Immunofluorescence images were captured using a ZEISS Axio Scope A1 upright microscope. The primary antibodies used were EDNRB (Proteintech, Rosemont, IL, USA; Cat#20964-1-AP), S100B (Proteintech, Cat#15146-1-AP), MITF (Proteintech, Cat#83803-2-RR), and PMEL (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cat#sc-377325). The cytoskeleton was labeled with Actin-Tracker Red-594 (Beyotime, Jiangsu, China; Cat#C2205S). To analyze target protein expression, three fields of view were randomly selected from each slide for analysis. Pixel-level fluorescence intensity was quantified using ImageJ v1.52, and the average fluorescence intensity per region was calculated to assess differences between groups.

Cell Lines and Cell Culture

Human UM cell lines 92.1 and Mel290 were kindly provided by Professor Martine J. Jager (Leiden University Medical Center, Leiden, the Netherlands). All cell lines were authenticated by short tandem repeat profiling. Cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco) at 37°C in a humidified incubator with 5% CO₂.

Lentiviral Infection

Knockdown cell lines were established via lentiviral transduction. The EDNRB short hairpin RNA sequences were designed, synthesized, and cloned into the pGLV vector. For lentivirus packaging, HEK293T cells were cotransfected with psPAX2, pMD2.G, and the short hairpin RNA-expressing plasmid using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) in Opti-MEM I reduced serum medium (Gibco). After 6 to 8 hours, the medium was replaced with fresh medium. Viral supernatants were harvested 48 hours post transfection and added to the target cells at an optimal multiplicity of infection.

RNA Isolation and Quantitative RT-PCR

Total RNA was extracted from cells using the EZBioscience Kit according to the manufacturer's instructions. Total RNA was reversely transcribed into cDNA with the PrimeScript RT reagent kit (Takara Bio, San Jose, CA, USA). Quantitative PCR was conducted on a LightCycler PCR system (Roche, Basel, Switzerland) using SYBR Green Master Mix (Vazyme, Nanjing, China). All reactions were run in triplicate. Gene expression was normalized to human ACTB, and relative levels of mRNA were calculated using the $2^{-\Delta\Delta CT}$ method. Primer sequences are listed in Supplementary Table S1.

Western Blot Analysis

For protein expression analysis, cells were lysed using RIPA lysis buffer (NCM Biotech, Newport, RI, USA) on ice, and the protein concentrations were measured by BCA Protein Assay Kit (Beyotime). Samples were separated by 7.5% (wt/vol) SDS-PAGE and transferred to PVDF membranes (Millipore, Burlington, MA, USA). Membranes were blocked with 5% nonfat milk and incubated with primary antibodies at 4°C overnight. After incubation with the appropriate fluorescent secondary antibodies (CST) for 1 hour at room temperature, the signals were detected with an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA). The primary antibodies used were EDNRB (Invitrogen, Cat#MA5-57023), NDUFB4 (Proteintech, Cat#27931-1-AP), UQCRC2 (Proteintech, Cat#14742-1-AP), and GAPDH (Proteintech, Cat#60004-1-Ig).

Bioenergetic Analysis

The oxygen consumption rate and extracellular acidification rate were measured using an XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Chicopee, MA, USA). One day before the assay, cells were seeded in XF96 cell culture microplates at a density of 10,000 cells per well. On the day of the experiment, the culture medium was replaced with either XF Glycolysis Stress Test Assay Medium or XF Cell Mito Stress Test Assay Medium, and cells were incubated for 1 hour at 37°C in a CO₂-free incubator to allow pH and temperature equilibration. For the glycolysis stress test, glucose (10 mM), oligomycin (2 μ M), and 2-deoxy-D-glucose (50 mM) were sequentially added. For the mitochondrial stress test, oligomycin (2 μ M), FCCP (1 μ M), and a mixture of antimycin A and rotenone (0.5 μ M each) were sequentially added. The oxygen consumption rate and extracellular acidification rate measurements were normalized to cell number post assay.

Statistical Analyses

Statistical analyses were conducted using GraphPad Prism version 9.2. All experiments were independently repeated three times to ensure reproducibility. Data were analyzed using two-tailed Student's *t* tests or one-way ANOVA, as appropriate. Results are presented as mean $\pm$ SEM, and a *P* value of less than 0.05 was considered statistically significant.

RESULTS

ClinicoPathological Characteristics of Enrolled Patients

We reviewed available FFPE tumor samples from patients diagnosed with UM who underwent enucleation between August 2022 and August 2024. After comprehensive clinico-pathological data collection, four patients were included in the study. Histopathological classification was confirmed by three experienced pathologists, identifying two patients with spindle cell UM (Spind_1 and Spind_2) and two with epithelioid cell UM (Epith_1 and Epith_2). None of the patients reported a family history of the disease. The average patient age was 48.5 years. All tumors were located in the choroid, with the largest basal diameter of approximately 18 mm

(Fig. 1A). Detailed clinical and pathological information is provided in Supplementary Table S2.

Representative MRIs from a spindle-type tumor (Spind_1; Fig. 1B) and an epithelioid-type tumor included in the analysis (Epith_2; Fig. 1C) are shown. In both cases, the tumor appeared moderately hyperintense on T1-weighted imaging and heterogeneously hypointense on T2-weighted imaging. Notably, exudative retinal detachment was present in both patients, likely owing to the large tumor volume.

Proteomic Profiling of Spindle and Epithelioid Tumors

To identify cell-type-specific proteomic signatures in UM, we performed regionally resolved proteomic analysis on archived FFPE enucleation samples. The experimental workflow is illustrated in Figure 2A. Briefly, after hematoxylin and eosin staining, two tumor-rich regions per sample were selected and validated by two pathologists to ensure maximal tumor purity and minimal contamination by stromal or microenvironmental cells. Selected areas were then microdissected, followed by protein extraction and liquid chromatography-tandem mass spectrometry analysis, following previous protocols.²⁰ The dissected spindle regions were labeled Spind_1_1, Spind_1_2, Spind_2_1, Spind_2_2, and epithelioid regions were labeled Epith_1_1, Epith_1_2, Epith_2_1, Epith_2_2 (Figs. 2B, 2C).

To ensure high-quality proteomic data, we performed stringent quality control. Protein intensity distributions across samples were highly consistent (Fig. 3A). Peptide length distributions conformed to expectations based on enzymatic digestion and fragmentation. Principal component analysis revealed clear clustering by cell type, underscoring the proteomic divergence between spindle and epithelioid tumors (Supplementary Figs. S1A, S1B).

Loss of Melanocyte Differentiation Markers in Epithelioid UM

Differential protein analysis revealed 217 proteins upregulated and 156 downregulated in epithelioid tumors relative to spindle tumors, indicating marked molecular differences (Fig. 3B). Notably, S100B, a key melanocyte differentiation marker, emerged as the most downregulated protein in the epithelioid group (Epith/Spind ratio = 0.12; *P* = 0.007) (Fig. 3C). To delve deeper into this observation, we analyzed the differentially expressed proteins between the two groups, with a particular focus on those associated with melanocyte differentiation. We found that EDNRB, a critical regulator of melanocyte stem cell fate, was also significantly downregulated in epithelioid tumors (Epith/Spind ratio = 0.57; *P* = 0.03) (Supplementary Table S3), supporting a dedifferentiation phenotype. A comprehensive comparison of other canonical melanocyte differentiation markers, including MITF, tyrosinase, tyrosinase-related protein 1, PMEL, and MLANA, is provided in Supplementary Table S4, illustrating a broad, albeit variable, trend of lineage repression in the epithelioid subtype. Furthermore, gene set enrichment pathway enrichment analysis highlighted elevated activity in cytoskeleton regulation pathway in spindle tumors (NES_Spind vs. Epith = 3.65; adjusted *P* = 0.05) (Fig. 3D), consistent with the morphological transformation

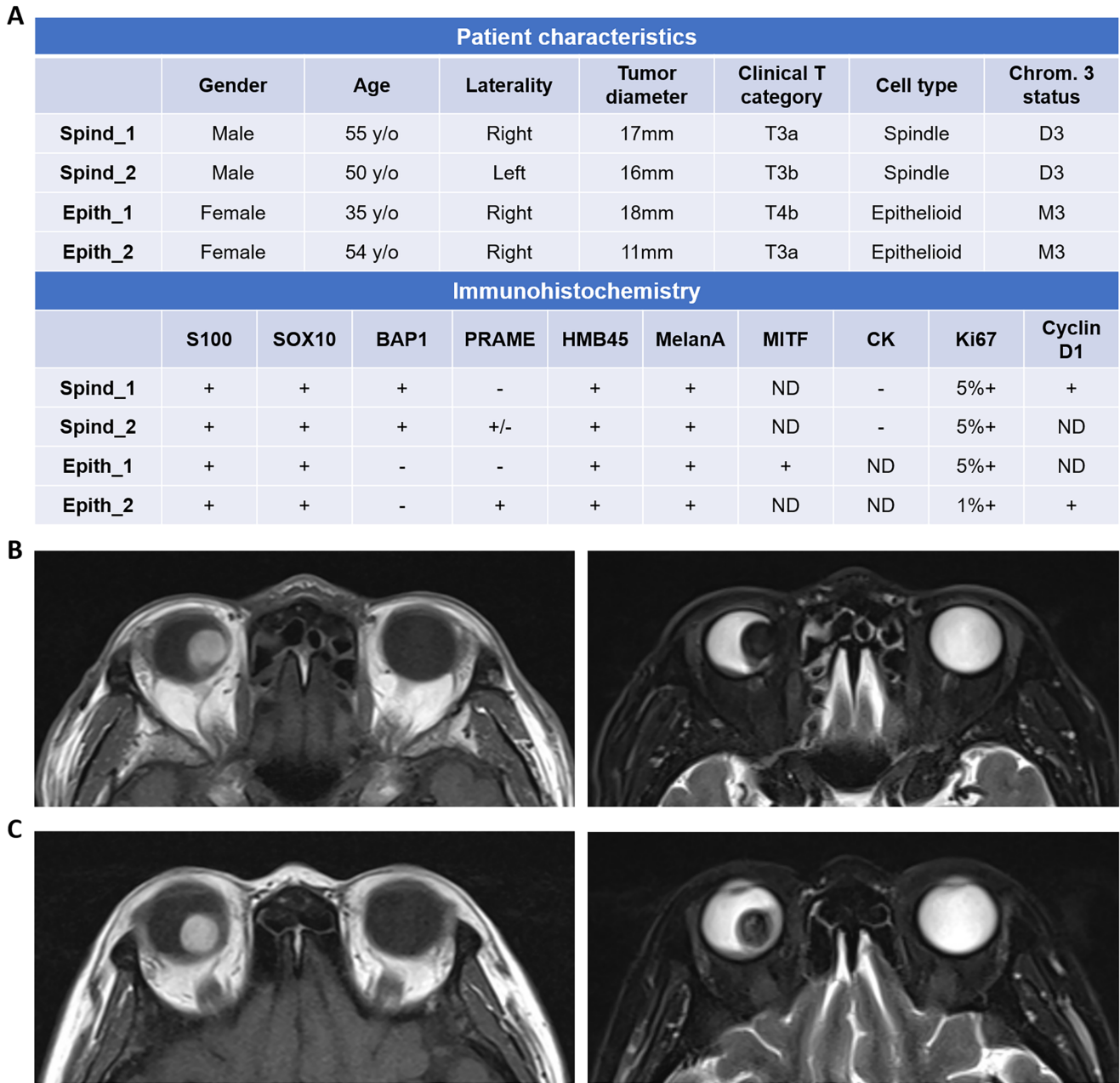


FIGURE 1. Clinicopathological characteristics of enrolled patients. (A) Summary of clinicopathological information for the included patients. D3, disomy 3; M3, monosomy 3; ND, not done. (B) Representative magnetic resonance imaging (MRI) from a spindle-type UM patient included in the analysis (Spind_1). (C) Representative MRI from an epithelioid-type UM patient included in the analysis (Epith_2).

from a rounded precursor to a polarized dendritic structure during melanocyte maturation.

To validate these findings, we performed immunofluorescence staining on the specimens ($n = 2$ per group). The results confirmed significantly lower expression of S100B and EDNRB in epithelioid compared with spindle tumor sections (Figs. 4A, 4B). Moreover, additional melanocytic markers MITF and PMEL also showed decreased expression in the epithelioid group, supporting reduced melanogenesis and melanosome maturation, respectively (Figs. 4C, 4D). These findings collectively suggest that a dedifferentiation process may underlie the transformation to the epithelioid UM phenotype.

EDNRB Inhibition Impairs Melanocytic Differentiation and Induces Epithelioid-Like Morphology in Spindle UM Cells

Because epithelioid UM exhibits reduced EDNRB expression and given EDNRB's critical role in regulating melanocyte stem cell fate, we investigated its role in melanocytic differentiation using the UM cell line 92.1. We first silenced EDNRB expression by transfecting two distinct short hairpin RNAs, which resulted in a significant reduction of EDNRB at both mRNA and protein levels (Figs. 5A, 5B). Morphological changes were assessed by quantifying dendritic arborizations per cell after immunofluorescent cytoskeletal stain-

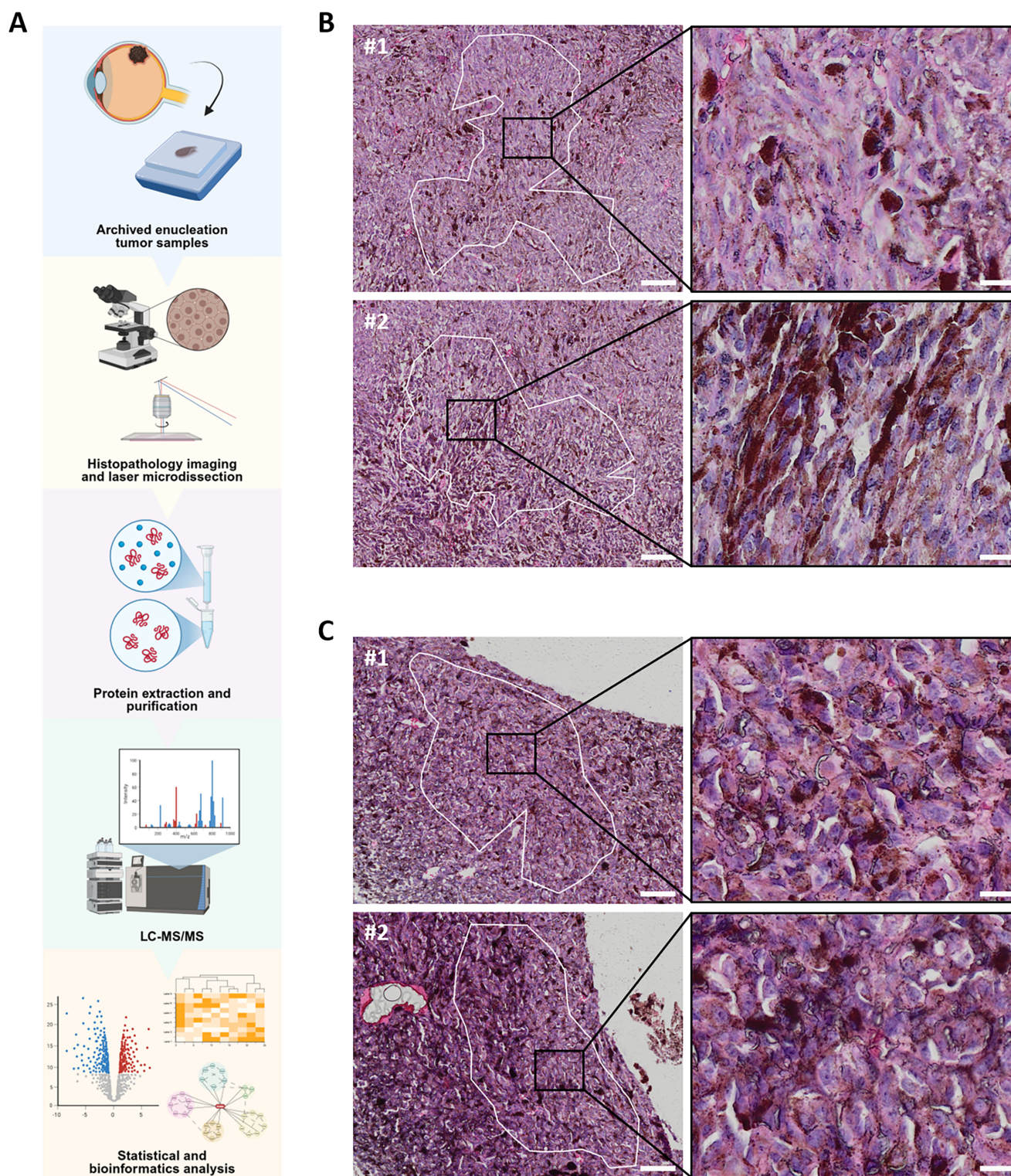


FIGURE 2. Workflow for proteomic profiling of microdissected regions. **(A)** Workflow diagram illustrating the microdissection-based proteomics sequencing process. **(B)** Representative hematoxylin and eosin staining of spindle UM samples at high magnification. Regions of interest are outlined in white ($n = 2$ per sample). Scale bars, 100 μm and 20 μm . **(C)** Representative hematoxylin and eosin staining of epithelioid UM samples at high magnification. Regions of interest are outlined in white ($n = 2$ per sample). Scale bars, 100 μm and 20 μm .

ing, with higher values indicating greater melanocytic differentiation (Fig. 5C). As expected, stable EDNRB depletion in 92.1 cells markedly reduced the number of dendritic arborizations compared with control cells (2.9 ± 0.7

and 2.7 ± 0.8 vs. 5.3 ± 0.9 dendritic processes per cell; $P < 0.0001$) and promoted a less polarized morphology resembling epithelioid UM cells (Fig. 5D). On the contrary, stable EDNRB depletion in epithelioid Mel290

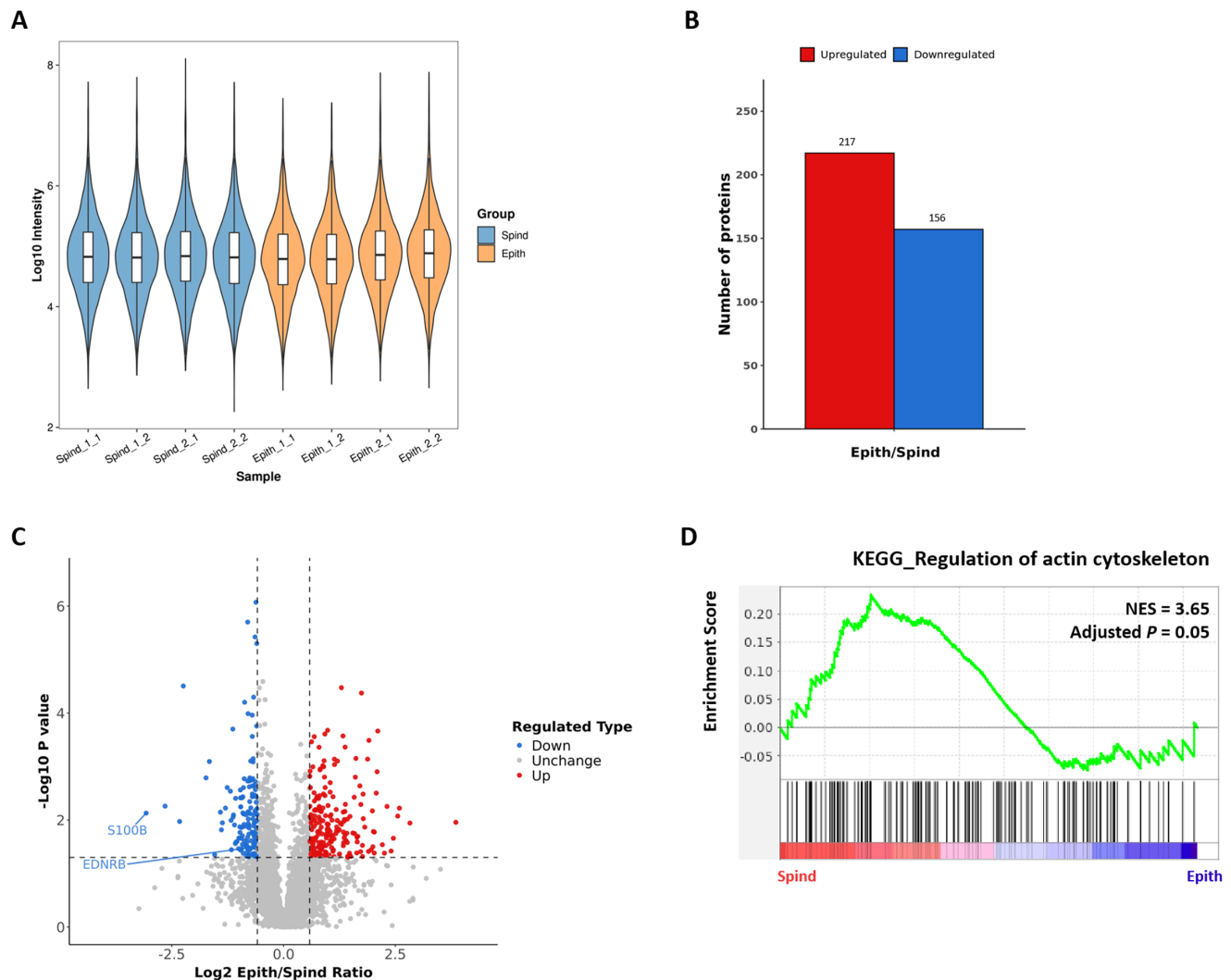


FIGURE 3. Microdissection-based proteomic analysis comparing spindle and epithelioid UM. **(A)** Violin plot showing quality control results for spindle and epithelioid samples ($n = 4$ per group). **(B)** Differentially upregulated and downregulated proteins in epithelioid vs. spindle samples. **(C)** Volcano plot showing upregulated (red), downregulated (blue), and unchanged (gray) proteins in epithelioid vs. spindle samples. **(D)** Gene set enrichment analysis enrichment analysis of cytoskeleton regulation pathways in spindle and epithelioid UM samples.

cells resulted in minimal changes to cell morphology compared with the 92.1 cell line (Supplementary Figs. S3A, S3B).

These findings suggest that EDNRB is indispensable for maintaining the spindle phenotype through its role in melanocytic differentiation. Loss of EDNRB disrupts the differentiation program, driving cells toward a less differentiated state. This finding is consistent with previous reports that EDNRB signaling is essential for melanocyte lineage commitment in melanocyte precursors.^{9,21} Importantly, such differentiation blockade may underlie the aggressive clinical behavior of epithelioid UM, because impaired differentiation has been linked to enhanced tumor plasticity and metastatic potential.^{22,23}

Epithelioid Tumors Display Enhanced Mitochondrial Respiration

Gene Ontology analysis revealed significant enrichment of metabolism-related terms in epithelioid vs. spindle tumor

samples (Supplementary Fig. S2A). Bubble plots indicated an upregulation of the biological processes involved in mitochondrial electron transport, adenosine triphosphate synthesis, and respiratory chain complex assembly (Fig. 6A; Supplementary Table S5). Further biological characterization using Kyoto Encyclopedia of Genes and Genomes enrichment analysis identified oxidative phosphorylation as the most significantly enriched pathway among differentially expressed proteins (Fig. 6B). In addition, single-sample gene set enrichment analysis confirmed that the oxidative phosphorylation pathway was consistently upregulated across all samples in the epithelioid group (NES_Spind vs. Epith = -14.20 ; adjusted $P = 0.02$) (Fig. 6C), suggesting a metabolic shift toward increased mitochondrial activity.

We next systematically examined alterations in the mitochondrial proteome by comparing the expression levels of respiratory chain complex proteins between the two histopathological UM subtypes. As shown in Figure 6D, key subunits of complexes I, III, and IV were markedly

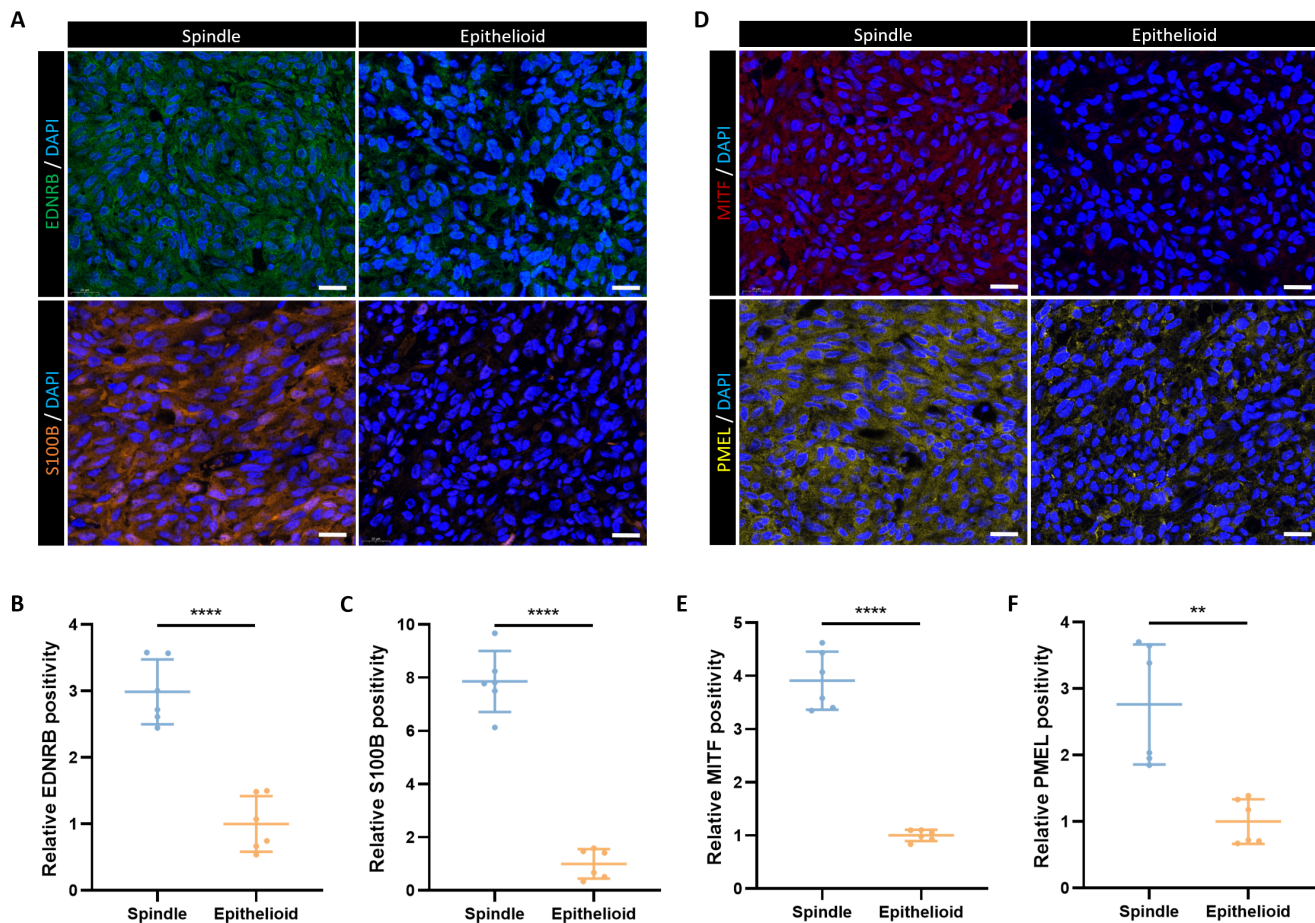


FIGURE 4. Immunofluorescence of melanocyte differentiation markers in spindle and epithelioid UM. (A) Representative immunofluorescence images showing EDNRB and S100B staining in spindle and epithelioid UM samples. Scale bar, 20 μ m. (B, C) Quantification of EDNRB and S100B positivity in spindle and epithelioid UM samples. Three fields per specimen were randomly selected for analysis. **** P < 0.0001. (D) Representative immunofluorescence images showing MITF and PMEL staining in spindle and epithelioid UM samples. Scale bar, 20 μ m. (E, F) Quantification of MITF and PMEL positivity in spindle and epithelioid UM samples. Three fields per specimen were randomly selected for analysis. ** P < 0.01.

elevated in epithelioid UM samples. This finding was consistent with WikiPathways enrichment analysis, which revealed significant enrichment in pathways such as electron transport chain OXPHOS system in mitochondria, mitochondrial complex I assembly model OXPHOS system, mitochondrial complex III assembly, oxidative phosphorylation, and mitochondrial complex IV assembly (Supplementary Fig. S2B).

Epithelioid UM Cell Line Shows Metabolic Reprogramming

To further validate the proteomics findings, we performed in vitro assays using two representative UM cell lines: 92.1 (spindle-like) and Mel290 (epithelioid). As shown in Figures 7A and 7B, cell morphology was assessed by the spindle morphology index (maximal cell length/width ratio) as previously reported, confirming distinct morphologies consistent with the histopathological subtypes.^{23,24}

We then focused on gene expression differences related to mitochondrial respiration. Among the OXPHOS-related proteins, those showing the most pronounced differences in the proteomic analysis included UQCRCQ, UQCRC1,

NDUFA12, UQCRB, NDUFB4, NDUFB8, UQCRC2, UQCRC10, and NDUFS5 (Fig. 7C; Supplementary Table S6). We next assessed the mRNA expression levels of these genes in 92.1 and Mel290 UM cell lines. The results confirmed elevated expression of most of these genes in Mel290 cells compared with 92.1, which is in alignment with the proteomics data (Fig. 7D). To further substantiate these findings at the protein level, Western blot analysis confirmed a significant upregulation of key respiratory chain subunits, NDUFB4 (complex I) and UQCRC2 (complex III), in Mel290 cells compared with 92.1 cells (Supplementary Figs. S4A, S4B).

Subsequently, we assessed mitochondrial respiration and glycolytic activity to characterize the bioenergetic profiles of the two cell lines. Mel290 cells exhibited a significantly higher oxygen consumption rate, along with increased basal and maximal respiration rates, indicating enhanced mitochondrial function. In contrast, glycolytic activity was reduced in Mel290 cells (Figs. 7E, 7F; Supplementary Figs. S5A, S5B). These results further support the notion that epithelioid UM cells undergo metabolic reprogramming, shifting from glycolysis toward increased reliance on mitochondrial respiration, consistent with the findings from proteomic profiling.

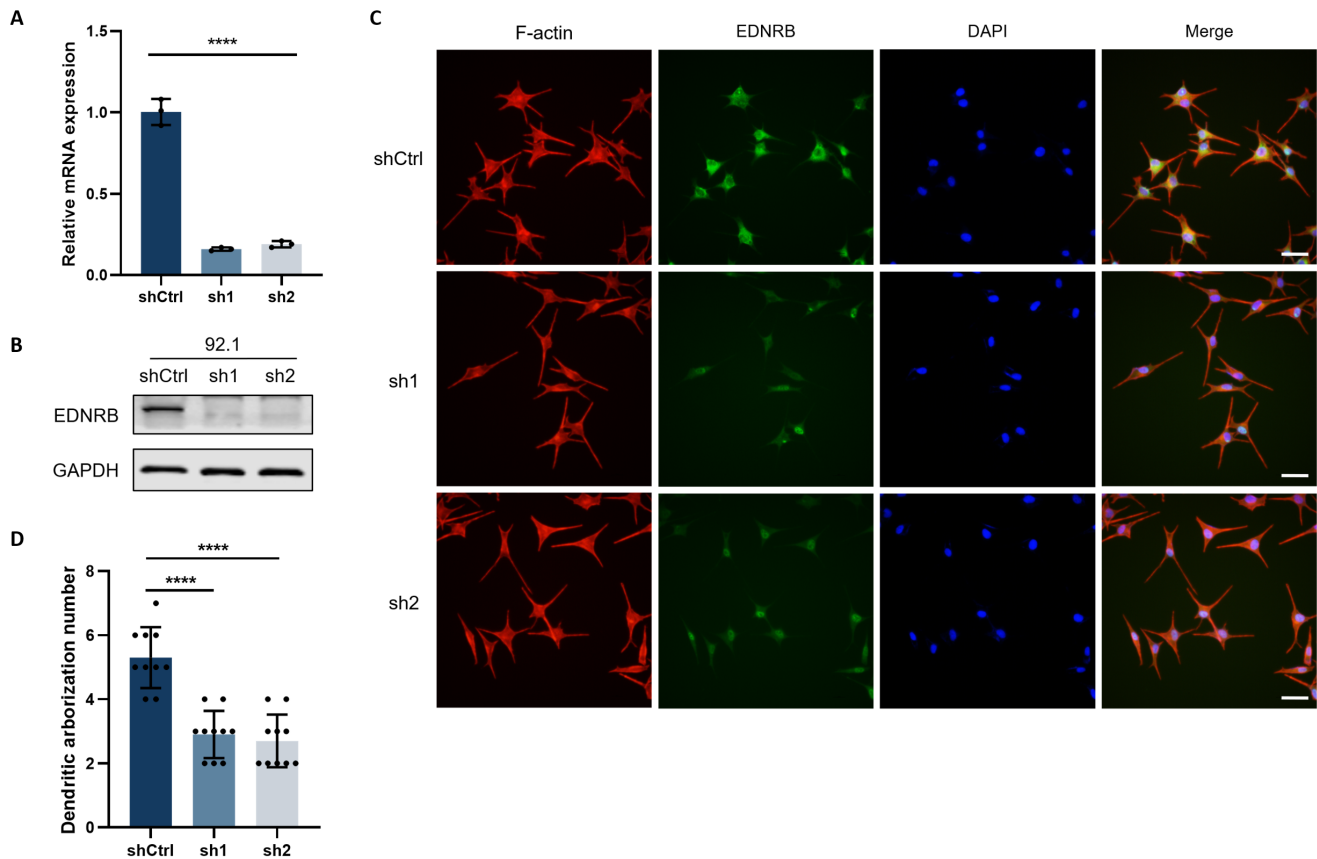


FIGURE 5. Loss of EDNRB induces a de-differentiated phenotype in UM cells. **(A)** Quantitative PCR data showed the decreased EDNRB expression upon knockdown. Data are presented as the mean $\pm$ SD of triplicate experiments. **** $P < 0.0001$. **(B)** Western blot analysis showed decreased protein levels of EDNRB after silencing EDNRB. GAPDH was used as the internal control. **(C)** Representative immunofluorescence images of F-actin (red), EDNRB (green), and DAPI (blue) in 92.1 cells. Scale bar, 50 μ m. **(D)** Mean number of dendritic arborizations in 92.1 cells upon EDNRB knockdown. **** $P < 0.0001$.

DISCUSSION

UM exhibits profound histopathological diversity, classically categorized into spindle, epithelioid, and mixed cell types based on morphological criteria established by Callender in 1931 and later refined by the Armed Forces Institute of Pathology.^{5,25} Spindle cells, characterized by elongated nuclei and cohesive growth patterns, are generally associated with a favorable prognosis, whereas epithelioid cells correlate with aggressive behavior, greater metastatic potential, and decreased survival.²⁶ Despite decades of clinical and pathological recognition, the proteomic underpinnings that distinguish these phenotypes remain poorly defined, limiting mechanistic insight into how tumor morphology influences clinical outcomes.

Previous studies have underscored molecular differences between UM subtypes. El-Shabrawi et al.²⁷ demonstrated elevated matrix metalloproteinase-9 expression in epithelioid compared with spindle UM using immunohistochemistry, correlating protein expression with histological subtype. Pérez-Pérez et al.²⁸ identified the PI3K/AKT/mTOR pathway mutations correlating with histological patterns, yet the bulk sequencing approach fell short of mapping the differences with cellular precision. LCM-based proteomics has emerged as a powerful tool to bridge morphological observations with molecular signatures by providing protein expression maps within precise tissue. In our

study, we leveraged this technology on FFPE tumor specimens to dissect molecular differences between histologically defined spindle and epithelioid regions. Our analysis revealed decreased expression of melanocytic differentiation markers alongside elevated OXPHOS protein expression specifically in epithelioid tumors, offering a refined perspective on the histopathological heterogeneity of UM (Fig. 8).

Our analysis generated the first high-resolution proteomic atlas of UM histopathological subtypes. We observed a striking loss of melanocyte differentiation markers in epithelioid tumors, with S100B emerging as the most markedly downregulated protein, followed by notable suppression of EDNRB. Reduced expression of MITF and PMEL in epithelioid regions, validated by immunofluorescence, further supports a dedifferentiation process in aggressive UM phenotype. S100B, a calcium-binding protein expressed in mature melanocytes, regulates cell cycle, differentiation, and survival, and has been widely used as a melanoma biomarker in both tissue and serum.¹⁰ Recent findings have shown that S100 expression is often lost in metastatic UM, potentially reflecting a shift toward a dedifferentiated state.²⁹ Similarly, the G-protein-coupled receptor EDNRB is essential for terminal melanocyte maturation, and prior studies have demonstrated that its reduced expression in primary UM is significantly associated with epithelioid morphology, as well as metastasis-related death.^{9,30} These



findings are consistent with broader observations linking reduced differentiation with enhanced malignancy in UM.^{23,31,32} Together, our results suggest that epithelioid transformation represents a loss of melanocytic lineage fidelity, favoring a more plastic, invasive, and clinically aggressive cell state.⁴

Building on these biological insights, the pronounced upregulation of mitochondrial respiration in epithelioid UM suggests a metabolic dependency that could be thera-

Although our study provides novel insights, several limitations warrant consideration. First, the small sample size, constrained by the availability of high-quality FFPE specimens, limits the generalizability of our findings. Future validation in larger, multi-institutional cohorts is essential. Second, regarding our *in vitro* validation, our choice of cell lines was further contextualized by recent multi-omics profiling of UM cell lines.⁴⁰ The study showed that, although 92.1 harbors a canonical *GNAQ* mutation and



retains melanocytic features, Mel290 is *GN4Q/11* wild type and genetically distinct from typical UM isolates. However, the proteomic analysis notably demonstrated that Mel290 is defined by a profound downregulation of melanosome-related proteins, clustering separately from differentiated UM lines. This independent dataset corroborates our observation that Mel290 represents a highly dedifferentiated state. Thus, whereas Mel290 may possess an atypical genetic background, its proteomic landscape makes it a valuable model for investigating the metabolic and functional consequences of the dedifferentiated, epithelioid phenotype observed in patient tissues. Future investigations would benefit from using an expanded panel of UM cell lines to further delineate the proteomic divergences between spindle and epithelioid phenotypes. Furthermore, our study design compared pure spindle and epithelioid regions

from distinct patients to maximize signal clarity. Although this approach effectively identifies fundamental proteomic differences between subtypes, it limits our ability to investigate the complex biological interactions or potential transitional states that might occur when these two cell populations coexist within a single mixed-type tumor. Additionally, although liquid chromatography-based proteomics offers robust regional resolution superior to bulk analysis, it fundamentally captures a regional average of the dissected tissue. Consequently, it lacks single-cell granularity and may mask finer-grained heterogeneity, such as rare subpopulations of cells with distinct metabolic states residing within the selected regions. Finally, functional experiments are needed to determine whether restoring differentiation or inhibiting OXPHOS can reverse aggressive traits in epithelioid cells, both *in vitro* and *in vivo*.

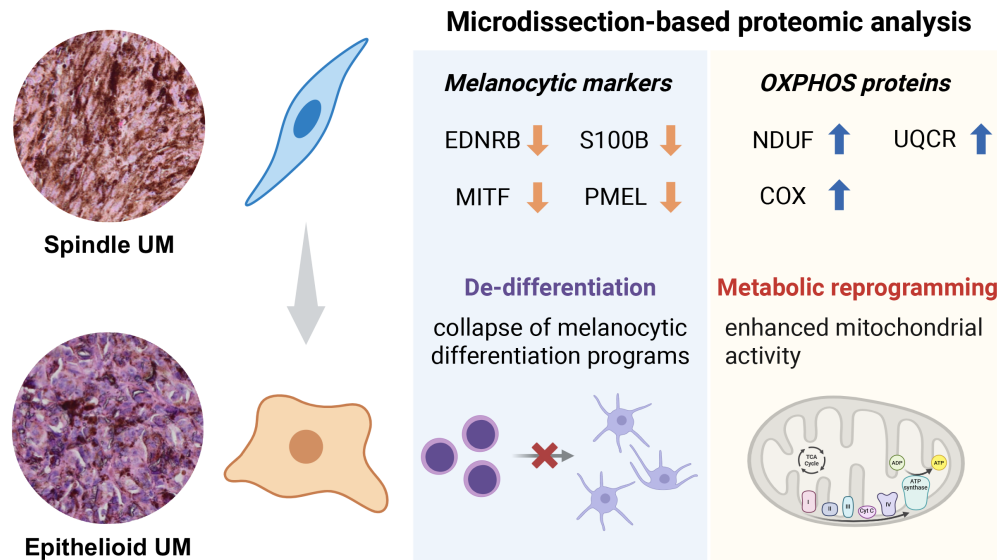


FIGURE 8. Mechanistic diagram illustrating molecular reprogramming in spindle and epithelioid UM cell types.

CONCLUSIONS

Our study provides the first regionally resolved proteomic landscape of UM histopathological heterogeneity, identifying epithelioid-specific dedifferentiation signatures and mitochondrial metabolic reprogramming. These findings deepen our understanding of UM biology and may inform future diagnostic and therapeutic strategies aimed at targeting differentiation state and metabolic vulnerabilities in aggressive UM.

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