TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Oxidative Phosphorylation Promotes Primary Melanoma Invasion

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Dermal invasion is a hallmark of malignant melanoma. Although the molecular alterations that drive the progression of primary melanoma to metastatic disease have been studied extensively, the early progression of noninvasive primary melanoma to an invasive state is poorly understood. To elucidate the mechanisms underlying the transition from radial to vertical growth, the first step in melanoma invasion, we developed a zebrafish melanoma model in which constitutive activation of ribosomal protein S6 kinase A1 drives tumor invasion. Transcriptomic analysis of ribosomal protein S6 kinase A1-activated tumors identified metabolic changes, including up-regulation of genes associated with oxidative phosphorylation. Vertical growth phase human melanoma cells show higher oxygen consumption and preferential utilization of glutamine compared to radial growth phase melanoma cells. Peroxisome proliferator activated receptor γ coactivator (PGC)-1 α , has been proposed as a master regulator of tumor oxidative phosphorylation. In human primary melanoma specimens, PGC1 α protein expression was found to be positively associated with increased tumor thickness and expression of the proliferative marker Ki-67 and the reactive oxygen species scavenger receptor class A member 3. PGC1 α depletion modulated cellular processes associated with primary melanoma growth and invasion, including oxidative stress. These results support a role for PGC1 α in mediating glutamine-driven oxidative phosphorylation to facilitate the invasive growth of primary melanoma. (*Am J Pathol* 2020, 190: 1108–1117; <https://doi.org/10.1016/j.ajpath.2020.01.012>)

The molecular mechanisms underlying melanoma metastasis have been studied extensively; however, the processes that control the early progression of primary melanoma are not well understood. During the radial growth phase (RGP), primary melanoma cells are confined to the epidermis. As the earliest step of progression, a melanoma transitions to a vertical growth phase (VGP), in which tumor cells invade the dermis and develop the capacity for metastasis.¹ This invasive behavior is a hallmark of melanoma and is thought to reflect its neural crest origin.² Some studies have identified molecular alterations that contribute to primary melanoma invasion. For example, deregulation of transcription factors, such as cyclic AMP-responsive element binding protein, leads to changes in expression or function of adhesion molecules, matrix-degrading enzymes, and survival factors that promote melanoma cell

invasion.³ Oncogenic activation of *BRAF* V600E in human melanocytes results in up-regulation of genes associated with growth, motility, inflammation, and extracellular matrix degradation.⁴ Multiple miRNAs, including miR-382 and miR-516b, have been shown to alter the expression of genes that are crucial for melanoma invasion and progression.⁵ However, to date no studies have reported a role for altered metabolism in the early progression of primary melanoma.

The importance of metabolic reprogramming in cancer initiation and progression is recognized,^{6,7} and melanoma

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cells are able to utilize and regulate multiple metabolic pathways to sustain their proliferation, invasion, and metastasis, including glycolysis, oxidative phosphorylation (OXPHOS), tricarboxylic acid cycle, and amino acid and lipid metabolism.⁸ Induction of the transcription coactivator peroxisome proliferator-activated receptor γ coactivator (PGC)-1 α (*PPARGC1A*) in a subset of metastatic melanomas by the melanocyte lineage factor microphthalmia-associated transcription factor (MITF) has been shown to promote mitochondrial capacity, OXPHOS, and resistance to oxidative stress and mitogen-activated protein kinase pathway inhibitors,^{9–11} but its relevance in primary melanoma remains to be established. Here, we tested the hypothesis that an altered metabolic state promotes invasive growth of primary melanoma, using an integrated approach that utilized a genetic zebrafish model of invasive melanoma, as well as human melanoma cell lines and patient tumor specimens.

Materials and Methods

Zebrafish Melanoma Model

To develop a zebrafish model of invasive melanoma, MiniCoopR vectors were generated as described.¹² An N-terminal myristoylation sequence (Myr-RSK1) was added to generate constitutively active human ribosomal protein S6 kinase A1 (RSK1; *RPS6KA1*).¹³ For melanoma assays, microinjection of zebrafish embryos was performed from an in-cross of Tg[*mitfa*:*BRAF*(V600E);*p53*(M214K/M214K);*nacre*]. Melanocyte-rescued animals were raised to adulthood and observed weekly for melanoma onset as previously described.¹⁴ Tumor invasion was assessed by histology, and melanoma tissue was used for RNA extraction for RNA sequencing (RNA-Seq).

Melanoma Cell Culture and RNA Interference

Primary melanoma cell lines (WM35, WM278, WM115, and WM3248) were obtained from the Wistar Institute (Philadelphia, PA), used within 20 passages after resuscitation, and cultured in MCDB153/L15 medium (4:1 v/v) supplemented with 2% fetal bovine serum, 5 mg/mL insulin, 15 mg/mL bovine pituitary extract, 1.68 mmol/L calcium chloride, 5 ng/mL epidermal growth factor, and 1% penicillin/streptomycin.

To knock down PGC1 α expression, siRNAs were purchased from Qiagen (Germantown, MD): *PPARGC1A* (PGC1 α ; catalog number SI02639833; AllStars negative control: catalog number 102780). WM3248 melanoma cells were transfected with siRNA against PGC1 α or with AllStars negative control siRNA at a final concentration of 30 nmol/L, using Lipofectamine 2000 reagent (Life Technologies, Grand Island, NY), according to the manufacturer's instructions.

RNA-Seq Analysis

Samples were sequenced at the Cornell University Transcriptional Regulation and Expression Facility (Ithaca, NY; zebrafish melanoma model) and the New York University Genome Technology Core (New York, NY) WM3248 human melanoma cells) using Hi-Seq. Per-read per-sample FASTQ files were generated using bcl2fastq2 Conversion software version 2.20 (Illumina, San Diego, CA) to convert per-cycle BCL base call file output by the sequencing instrument into the FASTQ format. Alignment software, STAR version 2.6.1d,¹⁵ was used for mapping reads to the hg19 human and Zv9 zebrafish reference genomes, respectively, and Fastq Screen software version 0.13.0 (Barbraham Institute, Cambridge, UK) was utilized to check for sample contamination. FeatureCounts software¹⁶ Subread package version 1.6.3 was used to generate matrices of read counts for annotated genomic features of each human and zebrafish data set. For differential gene statistical comparisons between groups of samples, the DESeq2 package¹⁷ Bioconductor version 3.10 in the R statistical programming environment was utilized. Genes with a false-discovery rate of <0.05 were considered significantly different between groups. Heatmaps were generated using the pheatmap R software package version 1.0.12 (Raivo Kolde, University of Tartu, Estonia). Pathway analysis was performed using ClusterProfiler version 3.14.3¹⁸ to identify enriched Kyoto Encyclopedia of Genes and Genomes signaling pathways based on top significantly expressed genes. Gene Set Enrichment Analysis in Python version 3.8 was used to identify genes that were enriched in a specific phenotype of interest. Additional gene list enrichment analysis of PGC1 α -regulated transcripts was performed using the EnrichR web server (<https://amp.pharm.mssm.edu/Enrichr>, last accessed December 17, 2019).¹⁹ RNA-Seq data were deposited at the NCBI Gene Expression Omnibus public repository (<https://www.ncbi.nlm.nih.gov/geo>, accession numbers GSE144117 and GSE145101).

Oxygen Consumption Rate Assay

Oxygen consumption rate (OCR) of human primary melanoma cell lines was assessed using the OCR Assay Kit (Cayman Chemicals, Ann Arbor, MI), according to the manufacturer's instructions. Antimycin A, a mitochondrial electron transport chain inhibitor, was used as a negative control for zero oxygen consumption. Fluorescence was measured using a SpectraMax M3 plate reader (Molecular Devices, LLC, San Jose, CA) at 380 nm (excitation) and 650 nm (emission). Oxygen consumption was calculated using linear regression to determine the slope for each signal profile.

Metabolic Profiling

Metabolic phenotype profiling of 367 carbon energy and nitrogen substrates was performed in WM35 and WM3248

primary melanoma cell lines using Phenotype MicroArrays M1 to M4 (Biolog, Hayward, CA), according to the manufacturer's instructions. Briefly, 2×10^4 cells were seeded in each well of four 96-well plates. After 48 hours of incubation at 37°C in 5% CO₂, Biolog Redox Dye MA (Biolog) was added to wells. Tetrazolium dye reduction for each well was determined using a SpectraMax M3 plate reader (Molecular Devices) at an absorbance of 590 nm.

Melanoma Patients and Tumor Tissue Specimens

Patients with primary melanoma ($n = 46$) were enrolled in the New York University Langone Medical Center Interdisciplinary Melanoma Cooperative Group biospecimen database, with prospectively recorded demographic, clinical, and pathologic data.²⁰ The study protocol was approved by the New York University School of Medicine institutional review board. All patients gave written, informed consent before the study.

PGC1 α , Ki-67, and SCARA3 IHC

Immunohistochemistry (IHC) was performed on formalin-fixed paraffin-embedded primary melanoma specimens, as described.²¹ Antibodies used were anti-PGC1 α rabbit monoclonal antibody (catalog number ab84139, 1:200 dilution; Abcam, Cambridge, MA), anti-Ki-67 rabbit monoclonal antibody D2H10 (catalog number 9207, 1:800 dilution; Cell Signaling Technology, Danvers, MA), or anti-scavenger receptor class A, member 3 (SCARA3) rabbit polyclonal N2C2 (catalog number GTX100595, 1:500 dilution; GeneTex, Irvine, CA). PGC1 α expression was scored based on staining intensity and distribution in tumors²² by an attending melanoma pathologist (F.D.) who was blinded to the clinical data. A score of 0 (absent), 1 (weak), or 2 (strong) was assigned to each sample based on PGC1 α staining intensity, compared to positive and negative control tissues. Next, the distribution of PGC1 α staining was assessed, in which focal staining (1% to 50% of the tumor) was a score of 1 and diffuse staining (>50% of the tumor) was a score of 2. The final PGC1 α expression score was the sum of intensity and distribution scores, resulting in three discrete PGC1 α expression groups: 0, absent; 2, weak; and 3, 4, strong. Ki-67 expression was scored as a percentage of positive tumor cells, as described.²³

RNA Extraction and Quantitative RT-PCR

Total RNA was isolated from cells using a QIAshredder and RNeasy Plus Mini Kit (Qiagen), according to the manufacturer's instructions. For cDNA synthesis, 0.5 μ g of RNA was reverse-transcribed using a QuantiTect Reverse Transcription kit (Qiagen). Quantitative RT-PCR was performed using TaqMan gene expression assays (*PPARGC1A*, Hs00173304_m1; *I8S*, Hs03003631_g1), TaqMan Fast Advanced Master Mix, and the Applied Biosystems

StepOnePlus Real-Time PCR System (Life Technologies). All data were normalized to expression of the *I8S* housekeeping gene. Relative PGC1 α expression levels between samples were calculated using the $2^{-\Delta\Delta C_t}$ method.²⁴

Results

Up-Regulation of Oxidative Phosphorylation in Invasive Melanoma

To identify mediators of primary melanoma invasion, we developed a zebrafish tumor model [Tg(*mitfa*:B-*RAF*(V600E));*p53*(lf);*mitfa*(lf)] that allowed expression of constitutively active Myr-RSK1 in rescued melanocytes from a MiniCoopR *tol2*-based transposon vector.¹⁴ Previously, this model was used to show that activation of RSK1, a key downstream effector of ERK1/2 signaling,²⁵ promotes vertical invasion of melanoma.²⁶ To gain a more detailed understanding of the mechanisms underlying the invasive growth of primary melanoma, this investigation identified cellular processes that are altered between Myr-RSK1 and Green fluorescent protein (control) tumors in this *in vivo* model using RNA-Seq (Figure 1A). The analysis revealed 1249 transcripts whose expression was significantly different (adjusted $P < 0.05$) between Myr-RSK1 tumors and Green fluorescent protein control tumors (Figure 1B and Supplemental Table S1). Notably, analysis of differentially expressed genes between control and RSK1-activated tumors identified significant enrichment for gene ontology terms associated with metabolism, including *electron transport chain*, *oxidative phosphorylation*, and *fatty acid biosynthesis* (Figure 1C and Supplemental Tables S2 and S3). The analysis also identified enrichment of gene ontology terms that have been associated with melanoma development and progression (Figure 1C), including extracellular matrix reorganization, cell cycle progression, regulation of growth, calcium signaling, histone modifications, and RNA processing.^{27–30} Further analysis using Kyoto Encyclopedia of Genes and Genomes gene sets identified 51 genes belonging to the OXPHOS pathway that were up-regulated in Myr-RSK1 tumors relative to Green fluorescent protein control tumors (Figure 1D).

To establish a role for increased OXPHOS in invasive primary human melanoma, this study used a cell-based assay to assess the OCR in a panel of RGP and VGP human melanoma cell lines (WM35, WM278, WM115, and WM3248). In this assay, the signal from a phosphorescent oxygen probe was quenched by oxygen, resulting in a signal that was inversely proportional to the amount of oxygen present in the sample. The results showed that the OCR was significantly higher in more invasive WM3248 VGP melanoma cells compared to RGP and less invasive VGP melanoma cell lines (WM35, WM278, and WM115) (Figure 1E). Finally, to understand the utilization of carbon sources to fuel this increased OXPHOS, a metabolic

Figure 1 A zebrafish model identification of increased oxidative phosphorylation in invasive melanoma. **A:** Zebrafish melanoma model used for RNA-Seq comparison of low- and high-invasive melanoma. Six independent tumors from each experimental group [green fluorescent protein (GFP) control or constitutively active/myristoylated ribosomal protein S6 kinase (Myr-RSK)-1] were used for RNA-Seq comparative analysis. **B:** Heatmap showing 1249 genes differentially expressed between zebrafish melanomas expressing GFP (control) or Myr-RSK1. Red and green indicate up- and down-regulated genes, respectively. **C:** Gene ontology analysis showing enrichment of biological processes among genes differentially expressed between Myr-RSK1 and GFP data sets. **D:** Heatmap showing 51 genes from the oxidative phosphorylation Kyoto Encyclopedia of Genes and Genomes (KEGG) gene set that are significantly up-regulated in RSK1 tumors. Red and blue indicate up- and down-regulation, respectively. **E:** Oxygen consumption rate for radial growth phase (RGP) (WM35) and vertical growth phase (VGP) (WM278, WM115, and WM3248) melanoma cell lines of increasing invasive potential. **F:** Metabolic profiling assay comparing utilization of glutamine and arginine as carbon sources in RGP (WM35) versus VGP (WM3248) melanoma cells. Data are expressed as means $\pm$ SD. $n = 6$ per group (**B**). $^{***}P < 0.01$ versus all other groups (one-way analysis of variance). ECM, extracellular matrix; GO, gene ontology; nc, noncoding.

Figure 2 Associations between peroxisome proliferator–activated receptor γ coactivator (PGC)-1 α and primary melanoma thickness and Ki-67 expression. **A: Top panel:** PGC1 α expression in primary melanomas of 4 mm thickness. **Bottom panel:** Ki-67 expression according to PGC1 α expression (absent, weak, or strong). **B:** Representative immunohistochemistry photomicrographs showing nuclear expression of PGC1 α (left column) and Ki-67 (right column) in primary melanoma specimens, each of which is of the nodular melanoma histologic subtype. **Insets** show the **main image** at higher magnification. Data are expressed as means $\pm$ SD. $n = 46$ (**A**). $*P < 0.05$ (two-tailed unpaired t -test). Scale bars: 0.2 mm (**main images**); 0.1 mm (**insets**). Original magnification: $\times 10$ (**main images**); $\times 40$ (**insets**).

phenotype microarray was used to compare RGP (WM35) and high invasive VGP (WM3248) melanoma cell lines. WM3248 cells showed increased utilization of glutamine and arginine carbon sources relative to WM35 cells (Figure 1F), suggesting that glutamine and arginine metabolism might support OXPHOS and an invasive VGP melanoma phenotype. Taken together, these results suggest a role for increased OXPHOS in primary melanoma invasion.

Association between Expression of PGC1 α and Primary Melanoma Invasion and Proliferation

The transcriptional coactivator PGC1 α promotes mitochondrial biogenesis in various tissues to increase energy production through OXPHOS.³¹ More recently, PGC1 α was shown to promote OXPHOS in metastatic melanoma.^{10,11} Based on the results of the OCR assays in RGP and VGP

melanoma cell lines, which showed that OXPHOS was elevated in highly invasive cells in this study, it was hypothesized that PGC1 α expression would be positively associated with tumor thickness in primary melanoma specimens. To test this hypothesis, IHC was used to assess PGC1 α protein expression in a cohort of primary melanoma specimens ($n = 46$) (Supplemental Table S4). Nuclear expression of PGC1 α was determined using a composite scoring method that reflected staining intensity and distribution (Materials and Methods), and samples were stratified into groups using the following IHC scale: 0, absent PGC1 α expression; 2, weak PGC1 α expression; or 3 or 4, strong PGC1 α expression. The results showed that 18 of 46 tumors (39.1%) had absent nuclear expression of PGC1 α , 12 of 46 tumors (26.1%) had weak nuclear expression of PGC1 α , and 16 of 46 tumors (34.8%) had strong nuclear expression of PGC1 α . Nuclear expression of PGC1 α was significantly higher in thick, more invasive primary melanomas compared to thin, less invasive primary melanomas (Figure 2), and a significant positive correlation was found to exist between PGC1 α expression and tumor thickness (Pearson $r = 0.437$, $P = 0.0024$). In parallel, the expression of the cellular proliferation marker Ki-67 was assessed by IHC in the same primary melanoma tissue cohort. A significant positive association between PGC1 α and Ki-67 expression was found (Figure 2). All tumors shown in Figure 2 belonged to the nodular melanoma histologic subtype (Supplemental Figure S1). These results are consistent with a role for PGC1 α in promoting the vertical growth and invasion that are a hallmark of early melanoma progression, at least in part through the up-regulation of OXPHOS.

Associations between PGC1 α Regulation of Expression of Genes and Oxidative Stress, Growth, and Invasion of Primary Melanoma Cells

This study next sought to elucidate how PGC1 α promotes primary melanoma progression. RNA interference was used to deplete PGC1 α levels in invasive WM3248 primary melanoma cells, and transfection with siRNA targeting PGC1 α produced a $>60\%$ reduction in PGC1 α mRNA levels (Figure 3A). To identify transcripts regulated by PGC1 α in primary melanoma, RNA-Seq was performed using RNA extracted from WM3248 cells that were transfected with siRNA against PGC1 α or negative control siRNA (Figure 3B). Genes whose expression was significantly altered after PGC1 α knockdown (adjusted $P < 0.05$ and $\log_2$ fold-change ≥ 0.5 or ≤ -0.5) (Supplemental Table S5) were analyzed for functional enrichment among canonical pathways (Wikipathways). In PGC1 α -depleted WM3248 cells, this study identified up-regulation of oxidative stress, cell cycle control, DNA damage response, senescence and autophagy, and apoptosis; and down-regulation of focal adhesion/phosphatidylinositol 3-kinase–Akt signaling, matrix metalloproteinases, focal

Figure 3 Peroxisome proliferator-activated receptor γ coactivator (PGC)-1 α knockdown induction of transcriptional changes associated with oxidative stress, growth, and invasion in WM3248 primary melanoma cells. **A:** Knockdown of PGC1 α mRNA in WM3248 primary melanoma cells after transfection with siRNA against PGC1 α (siPGC1 α) compared with negative control siRNA (siNC). **B:** Heatmap for comparison of gene expression (by RNA-Seq) between WM3248 cells transfected with siPGC1 α or siNC. Red and blue indicate up- and down-regulated genes, respectively. **C** and **D:** Analyses of functional pathways (Wikipathways) enriched for genes that are up-regulated (**C**) or down-regulated (**D**) by PGC1 α knockdown in WM3248 melanoma cells. Data are expressed as means $\pm$ SD. * $P < 0.05$ (two-tailed unpaired t -test). PI3K, phosphatidylinositol 3-kinase; TGF, transforming growth factor.

adhesion, neural crest differentiation, and transforming growth factor β signaling (Figure 3, C and D, and Supplemental Tables S6 and S7). The observation that PGC1 α -regulated transcripts were enriched for genes involved in cell cycle control was interesting, given the positive association between PGC1 α and Ki-67 identified earlier in primary melanoma specimens (Figure 2).

To further substantiate the link between PGC1 α , OXPHOS, and oxidative stress in invasive primary melanoma, this study assessed the expression of SCARA3, a protein that depletes reactive oxygen species (ROS) to protect cells from oxidative stress.³² First, significantly higher expression of *scara3* in invasive, RSK1-activated zebrafish melanomas compared to control tumors was observed (Figure 4A). Next, SCARA3 protein expression by IHC was assessed in a subset of primary melanoma tissues ($n = 22$) that we had also stained for PGC1 α . The results showed co-localization and a positive association between

PGC1 α and SCARA3 levels in serial tissue sections of primary melanoma (Figure 4B). Together, these findings suggest that the activation of SCARA3 expression could represent an antioxidant response in some tumors that helps to mitigate ROS production and support the invasive growth of PGC1 α high/OXPHOS high primary melanoma.

Discussion

Although the effects of somatic mutations and aberrant cell signaling pathways on the transition of primary melanoma from radial to vertical growth have been previously studied,^{1–5} there has been a limited understanding of the contribution of altered tumor metabolism to the invasive growth of primary melanoma. The current analyses using a zebrafish model of invasive melanoma and human primary melanoma cell lines and patient tissues support an important

Figure 4 Co-expression of the reactive oxygen species scavenger, scavenger receptor class A member 3 (SCARA3), with peroxisome proliferator-activated receptor γ coactivator (PGC)-1 α in human primary melanoma specimens. **A:** Increased *scara3* mRNA expression in tumors from myristoylated ribosomal protein S6 kinase (Myr-RSK1) zebrafish melanomas relative to green fluorescent protein (GFP) (control) zebrafish tumors. **B:** Representative immunohistochemistry (IHC) photomicrographs showing focal co-localization of PGC1 α (left panel) and SCARA3 (right panel) expression in serial tissue sections from human primary melanoma. **Arrows** denote areas of PGC1 α and SCARA3 positive staining. Data are expressed as means $\pm$ SD. $n = 22$ cases analyzed (**B**). * $P < 0.05$. Scale bars = 0.2 mm. Original magnification, $\times 10$.

role for OXPHOS in primary melanoma invasive growth. These metabolic analyses identified increased OCR and utilization of glutamine by invasive primary melanoma cells. This study shows a role for PGC1 α in mediating this invasive phenotype, and that its protein expression is associated with increased primary melanoma thickness and Ki-67 expression. The suppression of PGC1 α expression in primary melanoma cells led to alterations in oxidative damage/stress, cell cycle control, focal adhesion, activity of matrix metalloproteinases, and transforming growth factor β signaling, among other pathways. The links between PGC1 α , OXPHOS, oxidative stress, and the progression of primary melanoma were further substantiated by an increased expression of the ROS scavenger *scara3* in invasive zebrafish tumors and its colocalization with PGC1 α in human primary melanoma specimens, which is consistent with an antioxidant response in these tumors.

This study used a zebrafish model with a MiniCoopR expression system that produced melanocyte-specific activation of RSK1 and *BRAF* V600E, in addition to loss of function of p53 and MITF, to investigate the processes underlying invasive melanoma growth.¹⁴ The biological relevance of this model relates to the observation that RSK1

is hyperactivated in highly invasive VGP melanomas compared to less invasive RGP melanomas, and that RSK1 inhibition suppresses melanoma cell migration, invasion, and proliferation.²⁶ Zebrafish melanoma models have been shown to consistently recapitulate the human disease, as zebrafish melanocytes are distributed throughout the basal layer of the epidermis in a fashion similar to that in human melanocytes, whereas melanocytes localize primarily to hair follicles in mice.³³ Zebrafish models have been used to identify novel melanoma drivers and suppressors in the context of tumor initiation and progression, and to visualize interactions between melanoma and the tumor microenvironment.³³ This study highlights the utility of this zebrafish model as a tool to study regulators of melanoma invasion. To substantiate these findings, parallel analyses of human primary melanoma cell lines and tumor specimens from melanoma patients were performed. Interestingly, the increased OXPHOS we observed in invasive RSK1-driven zebrafish melanomas was not accompanied by up-regulated PGC1 α expression, which likely reflects underlying biological differences between humans and zebrafish. Rather than identifying homologous genes, the current zebrafish model revealed aberrant OXPHOS as a pathway that is associated with invasive growth of primary melanoma, and which could be interrogated in more detail in the context of human melanoma.

This study identified a role for altered tumor cell metabolism, and particularly for OXPHOS, in primary melanoma invasion. Previous studies have shown that some melanomas demonstrate the Warburg effect and are reliant on glycolysis for energy production,⁸ such as that induced by *BRAF* activation,³⁴ whereas other melanomas rely on oxidative phosphorylation associated with increased PGC1 α expression.¹¹ Tumor cells can also utilize multiple energy sources, including pyruvate or fatty acids.³⁵ Glutamine is an alternative carbon source for melanoma cells, and feeds the tricarboxylic acid cycle as well as driving fatty acid biosynthesis.³⁶ In this context, glutamine-fueled mitochondrial OXPHOS is decoupled from glycolysis and promotes metabolic plasticity of tumor cells that is associated with metastasis and drug resistance. For example, the metabolic switch of some melanomas to OXPHOS has been linked to resistance to mitogen-activated protein kinase pathway inhibitors.^{9,37} Furthermore, a recent study showed that OXPHOS is enriched in melanoma brain metastases compared to extracranial metastases, and that growth of melanoma brain metastases could be suppressed by an OXPHOS inhibitor, IACS-010759.³⁸ The potential utility of this strategy in melanoma patients has been questioned, as such inhibitors might be expected to block OXPHOS not only in tumor cells but also in anti-tumor T cells. However, inhibition of OXPHOS does not appear to block effector T-cell function, and instead has been shown to sensitize melanoma xenografts to immune checkpoint inhibitors, possibly in part by blocking the activity of myeloid-derived suppressor cells.³⁹

A central finding of this study was that PGC1 α protein levels were associated with increased primary melanoma thickness, invasion, and proliferation, suggesting that PGC1 α is involved in metabolic rewiring to increase OXPHOS and ATP production and drive an invasive primary melanoma phenotype. This finding is supported by reports that high PGC1 α expression is associated with a worse prognosis in metastatic melanoma.^{10,11} In contrast, Luo et al⁴⁰ found that PGC1 α knockdown promoted invasion of metastatic melanoma cell lines and that PGC1 α levels were inversely correlated with vertical growth in specimens from patients with primary melanoma. That finding suggests that the impact of PGC1 α knockdown may be context specific. Additionally, Luo et al⁴⁰ identified reduced levels of *PGC1A* mRNA in VGP versus RGP melanoma microarray data sets, but did not verify this finding with PGC1 α protein. In contrast, the present study assessed, by IHC, PGC1 α protein levels in tissues from patients with primary melanoma, and PGC1 α protein levels are more likely to reflect the true functional impact of PGC1 α in early melanoma progression, particularly given reports that have highlighted discrepancies between mRNA and protein expression *in vivo*.^{41,42}

A consequence of a metabolic switch to OXPHOS in some melanomas is the increased production of ROS, which promotes oxidative stress, mitochondrial release of cytochrome C, and apoptosis.⁴³ Tumor cells with high levels of PGC1 α may thus be protected from ROS in a PGC1 α -dependent manner,¹¹ since PGC1 α inhibition has been correlated with elevated ROS generation and a hypoxia-inducible factor 1 α -mediated switch to glycolysis.⁴⁴ Moreover, increased apoptosis of PGC1 α -inhibited A375P melanoma cells can be reversed by treatment with antioxidants.¹¹ These findings also suggest that SCARA3 may be an important component of the antioxidant response in melanoma cells as they adapt to OXPHOS to sustain their growth and invasion during early melanoma progression. Further studies are needed to fully define the mechanisms by which PGC1 α mitigates the oxidative stress associated with ROS generation in the context of primary melanoma growth and invasion.

In conclusion, the current study data using a zebrafish melanoma model, as well as human primary melanoma cell lines and tumor tissues, support a role for PGC1 α in mediating a metabolic switch to glutamine-driven OXPHOS during early melanoma progression. PGC1 α is up-regulated in thick primary melanomas and drives melanoma cell invasion and proliferation by modulating the expression of genes involved in oxidative stress, cell cycle control, DNA damage response, apoptosis, focal adhesion, matrix metalloproteinases, phosphatidylinositol 3-kinase–Akt, and transforming growth factor β signaling. This observation that highly invasive primary melanoma cells utilize glutamine to a greater extent than less invasive primary melanoma cells suggests that they might also be vulnerable to targeting glutamine metabolism or uptake. Several different

therapeutic strategies have been proposed to exploit this preference.^{45,46} Ultimately, an understanding of the role of metabolic rewiring in primary melanoma invasion and growth is likely to provide insight into the biology underlying melanoma progression, and might highlight novel vulnerabilities that could be targeted to treat advanced disease.

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Author Contributions

A.S. identified human melanoma samples for analysis, contributed significantly to writing manuscript; A.J. performed data analysis and literature review; I.B. performed IHC and assisted with RNA extraction; A.I. performed manuscript preparation; F.D. reviewed IHC and scored tissues; Y.H. performed zebrafish model development; K.G. was the primary contributor to intellectual and written components of the manuscript, performed statistical analyses, and designed and conducted experiments; I.O., oversaw the melanoma biobank and performed manuscript preparation.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.01.012>.

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