MicroRNA-211 Regulates Oxidative Phosphorylation and Energy Metabolism in Human Vitiligo

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Vitiligo is a common chronic skin disorder characterized by loss of epidermal melanocytes and progressive depigmentation. Vitiligo has complex immune, genetic, environmental, and biochemical causes, but the exact molecular mechanisms of vitiligo development and progression, particularly those related to metabolic control, are poorly understood. In this study we characterized the human vitiligo cell line PIG3V and the normal human melanocyte line HEM-I by RNA sequencing, targeted metabolomics, and shotgun lipidomics. Melanocyte-enriched microRNA-211, a known metabolic switch in nonpigmented melanoma cells, was severely down-regulated in vitiligo cell line PIG3V and skin biopsy samples from vitiligo patients, whereas its predicted targets PARCCT1, RR2M, and TAOK1 were reciprocally up-regulated. microRNA-211 binds to PGC1-α 3’ untranslated region locus and represses it. Although mitochondrial numbers were constant, mitochondrial complexes I, II, and IV and respiratory responses were defective in vitiligo cells. Nanoparticle-coated microRNA-211 partially augmented the oxygen consumption rate in PIG3V cells. The lower oxygen consumption rate, changes in lipid and metabolite profiles, and increased reactive oxygen species production observed in vitiligo cells appear to be partly due to abnormal regulation of microRNA-211 and its target genes. These genes represent potential biomarkers and therapeutic targets in human vitiligo.

INTRODUCTION

Vitiligo is a common, chronic skin disorder characterized by loss of epidermal melanocytes and progressive depigmentation (Ezzedine et al., 2012; Le Poole et al., 1993). Vitiligo is classified into two main types, nonsegmental (generalized) or segmental; affects approximately 0.5% of the global population (Ezzedine et al., 2012); and is often disfiguring and distressing to patients, who can suffer high levels of psychological morbidity (Silverberg and Silverberg, 2013).

The pathoetiology of vitiligo is multifactorial and has genetic, immunological, and environmental components. There is strong epidemiological and genetic evidence implicating an autoimmune pathogenesis in vitiligo, and it is strongly associated with other immune disorders (Gey et al., 2013; Richmond et al., 2013; Sandoval-Cruz et al., 2011). Genome-wide analyses have shown a number of autoimmune susceptibility loci in vitiligo including in tyrosinase, a crucial enzyme involved in melanin production (Jin et al., 2010; Spritz, 2010). Intrinsic cellular factors like oxidative stress and reactive oxygen species (ROS) also participate in vitiligogenesis (Jimbow et al., 2001); for example, NRF2-antioxidant responsive element signaling is impaired in vitiliginous melanocytes, rendering them vulnerable to oxidative stress (He et al., 2017; Jian et al., 2011, 2014). However, the exact molecular mechanisms underlying vitiligo pathogenesis remain unknown.

MicroRNAs (miRNAs) are approximately 22-nucleotide, noncoding RNA molecules that usually bind to the 3’ untranslated region (3’ UTR) of target mRNAs to suppress gene expression (Bartel, 2004; Jonas and Izaurralde, 2015). miRNAs participate in various cellular processes including proliferation, signal transduction, metabolism, apoptosis, and immune responses (Kloosterman and Plasterk, 2006; Rebade and Akdis, 2013; Sonkoly et al., 2008a). Dysregulated miRNA expression is associated with the pathogenesis of various inflammatory skin disorders such as psoriasis, atopic dermatitis, and allergic contact dermatitis (Sonkoly et al., 2008b), and there is some evidence implicating miRNAs in vitiligo. Gene expression microarray studies have shown...
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aberrant miRNA expression in the skin and serum of patients with vitiligo (Mansuri et al., 2014; Shi et al., 2013; 2014), with up-regulation of miR-224-3p, miR-4712-3p, and miR-3940-5p in peripheral blood mononuclear cells (Wang et al., 2015); miR-25 up-regulated in serum (Shi et al., 2016); and miR-99b, miR-125-b, miR-155, and miR-199a-3p up-regulated in the skin (Sahmatova et al., 2016) of vitiligo patients. Little is known, however, about the exact role of miRNAs, their targets, and their mechanism of action in the pathophysiology of vitiligo.

We recently reported that miR-211 is highly expressed in human melanocytes and is severely reduced in nonpigmented melanoma cells (Mazar et al., 2010), acting as a metabolic switch and affecting growth, differentiation, and apoptosis pathways as observed in vitiligo (Wang et al., 2016). This prompted us to interrogate miR-211 expression and its downstream effects on metabolism in clinical samples and in vitro, hypothesizing that miR-211 contributes to the vitiligo phenotype. We show that miR-211 is lost in the vitiligo lesions and in the human vitiligo cell line PIG3V, whereas putative miR-211 target genes such as PPARC1A, RRM2, and TAO1 are highly up-regulated. PIG3V cells fail to mount normal respiratory responses, and it is not attributable to mitochondrial numbers alone, which is partially reversed by miR-211 overexpression. Finally, PIG3V cells show enhanced production of ROS and widespread alterations in metabolism that might explain the impaired respiratory function and oxidative imbalance.

RESULTS
Decreased miR-211 expression in vitiligo patients’ skin and vitiligo cell line PIG3V
Recent findings showing (i) abnormal miRNA expression in the skin and serum of patients with vitiligo (Mansuri et al., 2014; Shi et al., 2014; Shi et al., 2013); (ii) that miR-211 positively controls pigmentation by targeting TGFBR2, a negative regulator of melanogenic enzyme TYR and TYP1 (Dai et al., 2015); and (iii) that miR-211-mediated reprogramming of primary fibroblasts triggers elevated expression of proinflammatory genes, cell proliferation, and migration, leading to melanoma growth (Dror et al., 2016), along with (iv) our own recent findings that miR-211 acts as a metabolic switch in nonpigmented melanoma cells (Mazar et al., 2016), prompted us to examine the expression and putative function of miR-211 and its downstream effectors in vitiligo pathogenesis. We reasoned that because miRNAs are known participants in the stress response and autoimmunity in vitiligo, the most highly differentially expressed genes in vitiligo lesions may regulate or be regulated by miRNAs, and miR-211 would be represented in the top down-regulated transcripts.

miR-211 expression was analyzed in vitiligo lesions (n = 11) and healthy control samples (normal skin pool from five different individuals) by quantitative real-time—PCR (qRT-PCR). miR-211 was significantly down-regulated in 10 out of 11 of the biopsy samples taken from patients with vitiligo (P < 0.0001) compared with normal skin (Figure 1a). Furthermore, analysis of nonlesional, perilesional, and lesional tissues from three patients with vitiligo showed a gradual loss of miR-211 expression from nonlesional to lesional regions (Figure 1b). We went on to examine miR-211 expression in primary melanocytes (HEM-l) and an immortalized perilesional melanocyte line from vitiligo (PIG3V) by qRT-PCR to examine whether the in vitro model replicated human disease. miR-211 expression was almost undetectable in PIG3V cells (Figure 1c). Thus, miR-211 is down-regulated in vitiligo melanocytes and might participate in vitiligogenesis.

Global transcriptomic changes in primary human melanocytes and vitiligo cells
To identify and characterize the genes and pathways participating in vitiligogenesis, we performed RNA sequencing of HEM-l and PIG3V cells (Le Poole et al., 2000). Hierarchical clustering showed that PIG3V cells showed a distinct expression pattern compared with primary melanocytes (see Supplementary Figure S1a online). Listed are the top 20 up-regulated and top 20 down-regulated transcripts (P ≤ 0.001, false discovery rate < 0.05) (see Supplementary Tables S1a and b online). The most significantly differentially expressed genes in pigmentation pathways (see Supplementary Table S2 online), cell cycle (see Supplementary Table S3 online), and immune responses (see Supplementary Table S4 online) are listed.

TRPM1, a calcium permeable cation channel expressed in melanocytes (Oancea et al., 2009), was observed in the top 20 down-regulated transcripts in PIG3V cells compared with HEM-l cells (10-fold) (see Supplementary Table S1b). The intrinsic sequence (sixth intron) of TRPM1 is known to encode miR-211. In addition to absent miR-211 expression (Figure 1c), pigment production was almost absent in PIG3V vitiligo cells both visually (see Supplementary Figure S1b) and quantitatively (see Supplementary Figure S1c). Expression of major pigmentation pathway genes, including KIT, MITF, TYR, TYRP1, DCT, PMEL, MART-1/MLANA, and TRPM1, was reduced in PIG3V cells compared with HEM-l cells (see Supplementary Figure S1d). TGFBR2, a known miR-211 target (Dai et al., 2015), was up-regulated in PIG3V cells compared with HEM-l cells (see Supplementary Figure S1e). To exclude the possibility that immortalization and repeated passage in PIG3V cells was responsible for miR-211 expression and pigmentation, miR-211 and pigmentation pathway gene expression were examined in a pigment-producing immortalized melanocyte line, PIG1 (Le Poole et al., 1997) (see Supplementary Figure S2 online). Similar results were seen between PIG1 and PIG3V cells for miR-211 expression (see Supplementary Figure S2a), melanin content (see Supplementary Figure S2b), and pigmentation pathway gene expression (see Supplementary Figure S2c).

To establish whether the transcriptional differences seen in HEM-l and PIG3V cells were representative of changes occurring in the lesions of patients with vitiligo, we overlaid our RNA sequencing data with gene expression array data derived from biopsy samples taken from patients with vitiligo (Gene Expression Omnibus accession series GSE53148 (Rashighi et al., 2014)). Despite the clinical samples originating from active vitiligo lesions and therefore containing infiltrating lymphocytes, highly up- or down-regulated transcripts in the clinical samples were
similar to those seen in HEML and PIG3V cells, including melanocyte-specific genes such as MLANA and DCT (Table 1 and Supplementary Figure S3 online).

PGC1-α is highly up-regulated in vitiligo cells and is a direct miR-211 target

miRNAs are known to exert transcriptional and post-transcriptional control by binding to target transcripts (Bartel, 2004). Loss of miR-211 expression in vitiligo melanocytes prompted us to check in silico (TargetScan [Agarwal V et al., 2015]) for putative miR-211 targets that might contribute to vitiligo pathogenesis and progression. Of the targets identified, PPARGC1A/PGC1-α, a transcriptional co-activator that regulates energy metabolism by acting as a master regulator of mitochondrial biogenesis (Finck and Kelly, 2006), was a putative target and was 2.4-fold up-regulated in PIG3V cells compared with HEM-I cells by RNA sequencing (see Supplementary Table S1a). Further, confirmatory qRT-PCR (Figure 2a), Western blotting (Figure 2b), and immunofluorescence (Figure 2c) showed significant up-regulation of PGC1-α in PIG3V cells compared with HEM-I cells. The predicted miR-211 target-binding site is located within the 3’ UTR region of PPARGC1A/PGC1-α (Supplementary Figure S4a online). To directly confirm that PPARGC1A is a miR-211 target, we cloned the 3’ UTR region of the PPARGC1A transcript with and without the miR-211 seed sequence to a luciferase reporter plasmid (PGC1-α-3’UTR and PGC1-α-3’UTR-miR211-del) followed by transfection into HEK293 cells. There was no difference in reporter gene expression between PGC1-α-3’UTR and PGC1-α-3’UTR-miR211-del transfected cells (see Supplementary Figure S4b online). However, when miR-211 was overexpressed, reporter activity was significantly reduced in PGC1-α-3’UTR transfected cells but not PGC1-α-3’UTR-miR211-del transfected cells (see Supplementary Figure S4b), confirming that miR-211 targets the 3’ UTR region in PGC1-α.

Cerium oxide nanoparticles (CNPs) have recently been used therapeutically in pathologies associated with chronic oxidative stress and inflammation because of their regenerative antioxidant property (Das et al., 2013). Transient overexpression using miR-211-coated CNPs (miR-211-CNP) led to a dramatic increase (>500-fold) in miR-211 levels in PIG3V cells (Figure 2d, left panel) and reciprocal down-regulation (>50%) of PGC1-α expression, as expected (Figure 2d, right panel). Furthermore, miR-211-CNP significantly reduced luciferase reporter activity in PIG3V cells transfected with PGC1-α-3’UTR but not PGC1-α-3’UTR-miR211-del plasmids (Figure 2e), further confirming that...
miR-211 targets PGC1-α in PIG3V cells. Finally, western blot analysis in PIG3V cells stably expressing miR-211 also showed a significant decrease in PGC1-α protein levels (Figure 2f).

We further validated PGC1-α expression in vitiligo lesions (n = 11) and healthy control samples by qRT-PCR. Consistent with findings in PIG3V cells, PGC1-α expression was significantly (P < 0.0001) up-regulated in 7 of 11 patient samples compared with normal (Figure 3a). Furthermore, RRM2 and TAOK1, which also contain miR-211 binding sites in their 3′ UTR region, were up-regulated in 90% (10/11) samples (Figure 3b and c). Reciprocal to miR-211 expression (Figure 1b), we observed a small but significant increase in PGC1-α and RRM2 but not TAOK1 expression in perilesional and lesional areas compared with nonlesional areas in 2 of 3 patients (see Supplementary Figure S5a online). Similar to PGC1-α, there was also a significant decrease in both RRM2 (20%) and TAOK1 (40%) gene expression in PIG3V cells transiently overexpressing miR-211, further supporting the hypothesis that miR-211 directly regulates their expression in vitiligo lesions (see Supplementary Figure S5b).

Defective respiration and mitochondrial complexes in vitiligo cells
PGC1-α is the main member of a family of transcriptional co-activators central to metabolic control, particularly mitochondrial biogenesis, adaptive thermogenesis, and glucco-neogenesis (Austin and St-Pierre, 2012; Handschin and Spiegelman, 2006). Reasoning that increased PGC1-α expression would increase the capacity of vitiligo cells to use oxygen, we first examined oxygen consumption rates (OCRs) using an extracellular metabolic flux analyzer. Surprisingly, the OCR of PIG3V cells was significantly lower (<50 pmol/minute) than in HEM-I (150 pmol/minute) cells (Figure 4a). Oligomycin, an adenosine triphosphate synthesis/electron transport inhibitor, reduced oxygen consumption in both cell types, suggesting that oxidative phosphorylation is active in these cell lines. However, when treated with trifluorocarbonylcyanide phenylhydrazone, an uncoupler of electron transport and oxidative phosphorylation, the OCR increased to over 300 pmol/minute in HEM-I cells compared with 90 pmol/minute in PIG3V cells, suggesting that PIG3V vitiligo cells have little capacity to respond to respiratory stress. Furthermore, glycolysis, as measured by the extracellular acidification rate, was also lower in PIG3V cells compared with HEM-I cells, suggesting that glycolysis does not substitute the energy requirements in these cells (Figure 4b) and that there is an intrinsic respiratory defect in vitiligo cells.

This prompted us to examine whether the respiratory defect seen in vitiligo cells could be explained by a decrease in mitochondrial number or occurred as a result of defects in the mitochondrial complexes forming the electron transport chain. The lower OCRs and dysfunctional maximal respiratory response were not attributable to mitochondrial numbers, which were similar in HEM-I and PIG3V cells (Figure 4c and d). However, oxidative phosphorylation complexes (mitochondrial complexes I–V in the inner mitochondrial membrane that generate adenosine triphosphate) were decreased in PIG3V cells (Figure 4e, and see Supplementary Table S5 online), especially complexes I, II, and IV, compared with HEM-I cells. Further, treatment of PIG3V cells with miR-211-CNP resulted in a moderate but significant recovery in basal respiration rate (Figure 4f).

### Table 1. List of differentially expressed genes in PIG3V and vitiligo lesions of patients compared with primary melanocytes (HEM-I) and normal skin, respectively

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Description</th>
<th>PIG3V/HEM-I Log2FC</th>
<th>P-Value</th>
<th>Vitiligo Lesion/Normal Skin Log2FC</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL7R</td>
<td>Interleukin-7 receptor</td>
<td>15.2</td>
<td>&lt;0.0001</td>
<td>3.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>OAS1</td>
<td>2′,5′-oligoadenylate Synthetase</td>
<td>12.8</td>
<td>&lt;0.0001</td>
<td>3.4</td>
<td>0.0057</td>
</tr>
<tr>
<td>C3</td>
<td>Complement component 3</td>
<td>11.5</td>
<td>&lt;0.0001</td>
<td>2.6</td>
<td>0.0100</td>
</tr>
<tr>
<td>FOSL1</td>
<td>FOS-like antigen 1</td>
<td>8.1</td>
<td>&lt;0.0001</td>
<td>2.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RRM2</td>
<td>Ribonucleotide reductase regulatory subunit M2</td>
<td>7.7</td>
<td>&lt;0.0001</td>
<td>3.2</td>
<td>0.0014</td>
</tr>
<tr>
<td>CCL5</td>
<td>C-C motif chemokine ligand 5</td>
<td>4.3</td>
<td>&lt;0.0001</td>
<td>4.1</td>
<td>0.0007</td>
</tr>
<tr>
<td>TAO1K</td>
<td>TAO kinase 1</td>
<td>4.0</td>
<td>&lt;0.0001</td>
<td>3.0</td>
<td>0.0100</td>
</tr>
<tr>
<td>IGFBP6</td>
<td>Insulin-like growth factor binding protein 6</td>
<td>3.8</td>
<td>&lt;0.0001</td>
<td>2.6</td>
<td>0.0046</td>
</tr>
<tr>
<td>HIST1H2AI</td>
<td>Histone cluster 1, H2a1</td>
<td>3.6</td>
<td>0.0002</td>
<td>2.6</td>
<td>0.0038</td>
</tr>
<tr>
<td>HIST1H4A</td>
<td>Histone cluster 1, H4a</td>
<td>1.6</td>
<td>0.0094</td>
<td>2.6</td>
<td>0.0026</td>
</tr>
<tr>
<td>ZNF324B</td>
<td>Zinc finger protein 324B</td>
<td>−1.0</td>
<td>&lt;0.0001</td>
<td>−1.7</td>
<td>0.0073</td>
</tr>
<tr>
<td>LYPLA2</td>
<td>Lysoospholipase II</td>
<td>−1.4</td>
<td>&lt;0.0001</td>
<td>−2.2</td>
<td>0.0024</td>
</tr>
<tr>
<td>ZNF500</td>
<td>Zinc finger protein 500</td>
<td>−1.5</td>
<td>0.0002</td>
<td>−2.1</td>
<td>0.0066</td>
</tr>
<tr>
<td>PHLD1A</td>
<td>Pleckstrin homology-like domain family A member 1</td>
<td>−1.8</td>
<td>&lt;0.0001</td>
<td>−1.6</td>
<td>0.0047</td>
</tr>
<tr>
<td>JOSD2</td>
<td>Josephin domain containing 2</td>
<td>−2.3</td>
<td>&lt;0.0001</td>
<td>−2.7</td>
<td>0.0058</td>
</tr>
<tr>
<td>GANC</td>
<td>Glucosidase alpha, neutral C</td>
<td>−2.7</td>
<td>0.0094</td>
<td>−2.6</td>
<td>0.0050</td>
</tr>
<tr>
<td>SLC45A2</td>
<td>Solute carrier family 45 member 2</td>
<td>−10.1</td>
<td>&lt;0.0001</td>
<td>−3.0</td>
<td>0.0019</td>
</tr>
<tr>
<td>MLANA</td>
<td>Melan-A</td>
<td>−10.9</td>
<td>&lt;0.0001</td>
<td>−4.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BCAN</td>
<td>Brevican</td>
<td>−11.1</td>
<td>&lt;0.0001</td>
<td>−4.4</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Abbreviation: FC, fold change.
PIG3V cells also showed increased PGC1-α mRNA and protein levels and decreased OCR and oxidative phosphorylation complexes compared with PIG1 cells (see Supplementary Figures S6a and b online). Taken together, miR-211-mediated regulation of PGC1-α does not appear to result in mitochondrial biogenesis, but the respiratory stress response is impaired in PIG3V cells, which might be attributable to mitochondrial complex dysregulation.

**Higher ROS production in vitiligo cells**

As well as regulating mitochondrial biogenesis, PGC1-α also affects the capacity of mitochondria to generate ROS either by up-regulation of complex I and III subunits or, alternatively, via the detoxification of ROS (St-Pierre et al., 2006; Valle et al., 2005). A host of studies have shown that ROS are overproduced in the vitiliginous lesions (Xie et al., 2016). As expected, intracellular ROS levels were significantly higher in PIG3V cells compared with HEM-l cells (Figure 4g). We hypothesized that overexpression of PGC1-α might be at least in part related to ROS overproduction in PIG3V cells. Small interfering RNA knockdown of PGC1-α (Figure 4h, left panel) significantly decreased intracellular ROS levels to baseline (HEM-l) (Figure 4h, right panel), suggesting that PGC1-α directly influences ROS levels in vitiligo cells.
Although PGC1-α expression is known to positively regulate the expression of ROS-detoxifying enzymes, its high expression in PIG3V vitiligo cells was not related to higher expression of NRF2, the master transcriptional regulator of many phase II detoxification enzymes. NRF2 expression was markedly and significantly down-regulated in PIG3V cells (see Supplementary Figure S7 online). Therefore, although the ROS-antioxidant balance is severely disrupted in PIG3V vitiligo cells and decreased PGC1-α partially reverses oxidative stress, it does not appear to exert its effects either via mitochondrial biogenesis or via ROS detoxification.

Vitiligo cells have unique lipid and metabolite profiles

Given that vitiligo cells showed defective respiratory activity and features of oxidative stress, we next performed global lipidomics and metabolomics profiling of vitiligo in PIG3V cells. We were keen to establish whether low levels of important substrates were altered in vitiligo cells and might therefore contribute to the vitiligo phenotype in melanocytes.

Lipidomic analysis showed cardiolipin up-regulation in PIG3V cells compared with HEM-1 cells (Figure 5a, and see Supplementary Table S6 online). Given that mitochondrial DNA levels were similar between these cells, it was more likely that adenosine triphosphate production in PIG3V cells was inefficient, perhaps because of the increased fatty acid biosynthesis using tricarboxylic acid cycle-generated citric acid (see below) and loss of respiratory efficiency through uncoupling. The markedly higher phosphatidylglycerol levels in PIG3V cells compared with HEM-1 cells further supported the observation of increased cardiolipin biosynthesis in PIG3V cells, because phosphatidylglycerol is a key substrate for cardiolipin synthesis in mammalian systems (Figure 5a).

Reduced phosphatidic acid and diacylglycerol levels were, as expected, associated with increased triacylglycerol levels, because diacylglycerol is a substrate of triacylglycerol biosynthesis and is largely produced from phosphatidic acid (Coleman and Lee, 2004). Together with inefficient mitochondrial adenosine triphosphate production, these findings suggest increased de novo fatty acid synthesis and inappropriate energy storage in triacylglycerol in PIG3V cells for unknown reasons (Figure 5b), strongly supported by profoundly higher levels of 16:0, 16:1, 18:1, 20:1, and 20:2 fatty acyl chains in the triacylglycerol pool of PIG3V compared with HEM-1 cells without significant changes in polyunsaturated fatty acids, which are exogenously supplied (see Supplementary Figure S8a online). Phosphatidylyceroline, sphingomyelin, and phosphatidylserine are major cellular membrane components and play important roles in maintaining normal cellular functions. For example, sphingomyelin is a key component in lipid rafts (Lingwood and Simons, 2010), and phosphatidylserine is an important component of signal transduction and apoptosis (Tyurina et al., 2000). Marked changes in these lipids were observed in PIG3V compared with HEM-1 cells. Specifically, there was 2-fold higher phosphatidylcholine but half the amount of sphingomyelin and phosphatidylserine levels in PIG3V cells compared with HEM-1 cells (Figure 5c). The most reduced sphingomyelin species were N18:0 and N24:1 (see Supplementary Figure S8b), reduction of which indicates down-regulation of ceramide synthase 1, further supported by the ceramide mass levels (N18:0 and N24:1 ceramide lower in PIG3V than HEM-1 cells and N16:0 ceramide the same) (see Supplementary Figure S8c).

Finally, we performed targeted metabolomics in HEM-1 and PIG3V cells and looked at the significant changes associated with amino acids and organic acids in HEM-1 and PIG3V cells.

Figure 3. Expression of putative miR-211 target genes is increased in vitiligo lesions. Analysis of (a) PGC1-α, (b) RRM2, and (c) TAO1 in healthy skin (normal skin pool from five individuals) and vitiligo lesions (n = 11) by quantitative real-time PCR. Results shown are mean ± standard deviation of mean. Student t test was performed to detect differences in normal skin pool and individual patient samples. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. miRNA, microRNA; ns, not significant.
PIG3V cells (Figure 5d and e). Significant decreases in succinate, malate, and \(\alpha\)-ketoglutarate levels (Figure 5d) in PIG3V cells indicate multiple defects in mitochondrial tricarboxylic acid cycle intermediates. This corroborates our previous findings showing aberrant mitochondrial assembly and inefficient respiration in PIG3V cells compared with HEM-I cells (Figure 4a–e). Profiling of amino acids showed significant increases in alanine, arginine, glycine, and methionine in PIG3V cells, whereas citrulline and ornithine levels were dramatically decreased (Figure 5e), and modest decreases in serine and valine levels were also observed. The turnover of respiratory substrates might be more important than mitochondrial numbers in energy use in vitiligo cells. Further analysis of lipid and metabolite profiles in miR211-CNP–treated PIG3V cells could be helpful in understanding the role of miR-211 in vitiligo.

**DISCUSSION**

Here we report decreased levels of miR-211 in vitiliginous lesions and in PIG3V, an immortalized perilesional melanocyte cell line. Correspondingly, the putative miR-211 target genes \(PPARGC1A\) (PGC1-\(\alpha\)), \(RRM2\), and \(TAOK1\) were up-regulated in PIG3V cells and lesional biopsy samples. We show that miR-211 binds to and represses PGC1-\(\alpha\) at the 3’ UTR locus. PIG3V cells have abnormal respiratory responses and mitochondrial complex assembly, and reintroduction of miR-211 augmented oxygen consumption in affected melanocytes. This study represents a comprehensive lipidomic and metabolomic analysis of normal and vitiliginous melanocytes and suggests that the turnover of respiratory substrates might be more important than mitochondrial numbers in energy utilization in vitiligo cells.
miR-211 is expressed in primary melanocytes, where it influences various cellular processes including pigmentation. Its loss is implicated in melanomagenesis (Dror et al., 2016; Mazar et al., 2010, 2016), where it alters metabolism (Mazar et al., 2016). Similarly, here we observed loss of miR-211 expression in vitiligo lesions. Although this loss may be attributable to the loss or negligible numbers of melanocytes in these lesions because of autoimmunity (Alikhan et al., 2011; Ezzedine et al., 2015), it is unlikely that a single dominant pathway is responsible for melanocyte loss in vitiligo. Indeed, perilesional PIG3V cells also showed a significant decrease in miR-211 expression and pigmentation compared with both primary (HEM-1) melanocytes and the melanocyte line (PIG1), suggesting that miR-211 loss represents an intrinsic cellular defect in affected cells that contributes to vitiligo pathogenesis.

Figure 5. Lipidomic and metabolomic analysis of HEM-I and PIG3V cells. (a–c) Graphs depict cumulative changes in fatty acid chains for different lipid groups. (a) Cardiolipin and phosphatidylylycerol. (b) Phosphatidic acid, diacylglycerol, and triacylglycerol. (c) Phosphatidylcholine, sphingomyelin, and phosphatidylserine. (d, e) Graphs depict significant changes in (d) organic acids and (e) amino acids in HEM-I and PIG3V cell lysates. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Although not obtained from the same patient, HEM-I and PIG3V cells proved to be a good in vitro model of clinical vitiligo. Transcriptomic analysis of HEM-I and PIG3V showed similar changes in many of the immune, cell cycle, and pigmentation pathway genes shown to be differentially expressed in vitiliginous lesions (Dey-Rao and Sinha, 2016; Rashighi et al., 2014; Regazzetti et al., 2015). Our finding of highly significant down-regulation of pigment genes downstream of MITF (a master regulator of melanogenesis, 6-fold down-regulated) in PIG3V cells confirmed defects in melanin biosynthesis and pigmentation and provided confidence that the changes seen in vitro were representative of the clinical disease state.

Because miRNAs usually exert their effects by controlling the expression of target genes, understanding the role of miR-211 in vitiligoogenesis requires an understanding of its downstream effects. Candidate genes such as PPARGC1A, RRM2, and TAOK1 were up-regulated in vitiligo lesions and also had miR-211 binding sites in their 3’-UTR regions, making them putative miR-211 targets. Although PGC1-α is known to play a role in mitochondrial biogenesis, mitochondrial numbers, mitochondrial complex assembly proteins, and oxygen consumption rates were lower in PIG3V cells in our experiments. However, the inability of vitiligo melanocytes to mount normal respiratory responses shows a different aspect of vitiligo pathophysiology, and this disordered metabolism might contribute to melanocyte fragility and subsequent loss in vitiligo. The decreased oxygen consumption rates may not be solely due to decreases in mitochondrial numbers but rather alterations in their composition and assembly and the way in which they use available substrates.

In summary, our results suggest that miR-211 may play a crucial role in regulating the pathophysiology of human vitiligo, which is not only an immunological disorder but also a disease of abnormal cellular metabolism. miR-211 and its targets may play important roles in oxidative phosphorylation and mitochondrial energy metabolism in vitiligo and have therapeutic and biomarker potential. Ultimately, a thorough understanding of intracellular metabolism in vitiligo cells is required so that these pathways can be targeted for therapeutic purposes.

**MATERIALS AND METHODS**

**Patient samples**

This study received ethical approval from the Sanford Burnham Prebys Medical Discovery Institute Institutional Review Board Committee, and all participants signed written informed consent. miRNA expression analysis was conducted by qRT-PCR on 11 patients with vitiligo (7 men and 4 women; age range = 25–70 years) attending the Associates in Dermatology outpatient clinics (Orlando, Florida). The diagnosis of vitiligo was based on history, examination, and pathological findings. Nonlesional, perilesional, and lesional tissues were obtained from three patients with active nonsegmental vitiligo from Department of Dermatology and Pediatric Dermatology, National Reference Center for Rare Skin Disorders, Hôpital Saint-André, Bordeaux, France. RNA from human Adult Normal Tissue 5 Donor Pool: Skin (1 man and 4 women; age range = 65–83 years; catalog #R1234218-P; BioChain, Newark, CA) was used as control. None of the control subjects had any history of chronic skin disease or vitiligo in their families. The samples collected from patients with vitiligo were either shave or punch skin biopsy samples taken from the lesional and perilesional areas and were formalin fixed and paraffin embedded according to standard protocols.

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.04.025.

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