Repigmentation of Human Vitiligo Skin by NBUVB Is Controlled by Transcription of \( GLI1 \) and Activation of the \( \beta \)-Catenin Pathway in the Hair Follicle Bulge Stem Cells

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Vitiligo repigmentation is a complex process in which the melanocyte-depleted interfollicular epidermis is repopulated by melanocyte precursors from hair follicle bulge that proliferate, migrate, and differentiate into mature melanocytes on their way to the epidermis. The strongest stimulus for vitiligo repigmentation is narrow-band UVB (NBUVB), but how the hair follicle melanocyte precursors are activated by UV light has not been extensively studied. To better understand this process, we developed an application that combined laser capture microdissection and subsequent whole transcriptome RNA sequencing of hair follicle bulge melanocyte precursors and compared their gene signatures to that of regenerated mature epidermal melanocytes from NBUVB-treated vitiligo skin. Using this strategy, we found up-regulation of \( TNC, GJB6, \) and \( THBS1 \) in the hair follicle bulge melanocytes and of \( TYR \) in the epidermal melanocytes of the NBUVB-treated vitiligo skin. We validated these results by quantitative real-time–PCR using NBUVB-treated vitiligo skin and untreated normal skin. We also identified that \( GLI1 \), a candidate stem cell-associated gene, is significantly up-regulated in the hair follicle (HF) (Birlea et al., 2017a). The strongest stimulus for the activation of melanocyte precursors is narrow-band UVB (NBUVB), but this process has not yet been adequately studied in the HF. Our recent immunostaining study, using key functional markers, showed that the HF bulge of depigmented vitiligo skin is inhabited by melanocyte stem cells (DCT\((+)/C-KIT\((+)\)) and melanoblasts (DCT\((+)/C-KIT\((+)\)), which proliferate, migrate, and differentiate during NBUVB treatment on their way to epidermis (Goldstein et al., 2015). The proportion of melanocyte precursor populations slightly increased in the HF bulge after NBUVB treatment, indicating melanocyte activation. To more thoroughly characterize the repigmentation process, we developed an application that, to our knowledge, was previously unreported that combined laser capture microdissection of skin cells isolated from vitiligo patients with RNA-sequencing (RNA-seq). With this application, we tested the gene signature of the HF bulge (the source of melanocyte precursors) and of the IE (the repopulated site) in NBUVB-treated vitiligo skin. Our strategy consisted of a rapid immunostaining protocol, followed by fluorescent laser capture microdissection (F-LCM) of melanocytes and, separately, of keratinocytes from the HF bulge and epidermal basal layer. This was followed by RNA isolation, whole-transcriptome RNA-seq, and gene expression analysis. Our RNA-seq analysis identified the integrin pathway as the top pathway activated in the bulge melanocytes of NBUVB-treated vitiligo skin, compared with NBUVB-treated vitiligo epidermis, and \( \beta \)-catenin as the top

INTRODUCTION

Vitiligo is a common depigmentation disorder, affecting 0.3–0.5% of the population worldwide. It is characterized by white patches of the skin, due to autoimmune destruction of epidermal melanocytes by melanocyte-reactive cytotoxic T cells (Eby et al., 2014; Rashighi and Harris, 2017; Spritz, 2013). Vitiligo repigmentation is characterized by repopulation of melanocyte-depleted interfollicular epidermis (IE) with melanocyte precursors, mostly originating from the hair follicle (HF) (Birlea et al., 2017a).
upstream transcription regulator. We also found up-regulation of TNC, GJB6, and THBS1 in the bulge melanocytes and of TYR in the epidermal melanocytes of NBUVB-treated vitiligo skin, results validated by quantitative real-time PCR (qRT-PCR). We also identified that GLI1, a candidate stem cell-associated gene, is significantly up-regulated in melanocytes captured from the NBUVB-treated vitiligo bulge compared with the untreated vitiligo bulge. These signals and pathways may have regulatory roles in the activation of bulge melanocyte precursors during the vitiligo repigmentation process.

**RESULTS**

We used frozen biopsy samples from untreated and NBUVB-treated vitiligo patients to perform bulge mapping, rapid immunostaining, F-LCM of specific skin cells, and RNA-seq analysis.

**Alignment and mapping of RNA-seq reads**

Whole-transcriptome RNA-seq, performed with a HiSeq 2000 sequencer (Illumina Inc., San Diego, CA), generated on average 3.2 and 3.6 million single-read sequences per sample from each studied site (HF bulge and IE, respectively), with an average length of 250 base pairs (bp) without adapters and of 325 bp with adapters. The median total raw reads for the bulge and IE samples were approximately 5.3 million and 5.9 million, respectively, and the median passing rates for the bulge and epidermis were 84.6% and 85.6%, respectively, indicating good data quality. We discarded samples that did not meet the control criteria because of quality, presence of contaminants formed by adapter-adapter ligation, and presence of reads without insert tags.

**RNA-seq gene expression profiles show cell-specific and site-specific F-LCM capture differences**

We isolated RNA from both melanocyte and keratinocyte samples. The purpose of including the keratinocytes was to show the specificity of our capture. After the validation step, we focused this study on the melanocyte material of the NBUVB-treated vitiligo skin. The total RNA from melanocyte samples and separately from keratinocyte samples was isolated by F-LCM from six NBUVB-treated vitiligo patients and subjected to RNA-seq followed by gene expression analysis. From the principal component analysis (PCA), we observed a good segregation of gene expression values between (i) the melanocytes captured from the NBUVB-treated vitiligo bulge versus the melanocytes from the NBUVB-treated vitiligo IE (see Supplementary Figure S1a online), (ii) the keratinocytes captured from the NBUVB-treated vitiligo bulge versus keratinocytes from the treated vitiligo IE (see Supplementary Figure S1b), (iii) the melanocytes versus keratinocytes captured from the NBUVB-treated vitiligo IE (see Supplementary Figure S1c), (iv) the melanocytes versus keratinocytes captured from the NBUVB-treated vitiligo bulge (see Supplementary Figure S1d). Additionally, we examined the expression of melanocyte-specific genes and keratinocyte-specific genes in our samples to determine cell specificity of capture (Figure 1a–d). We found significant enrichment of the melanocyte-specific genes in melanocytes compared with keratinocytes and significant enrichment of the keratinocyte-specific genes in keratinocytes compared with melanocytes in both NBUVB-treated vitiligo epidermis (Figure 1c) and NBUVB-treated vitiligo bulge (Figure 1d). Based on the results of principal component analysis and cell-type–specific gene expression analysis, we concluded that our F-LCM method is effective and accurate for selectively isolating RNA material from distinct populations of melanocytes and adjacent keratinocytes.

**RNA-seq shows differentially expressed genes in the melanocyte samples of the HF bulge compared with epidermis of the NBUVB-treated vitiligo skin**

Next, we focused our analysis on characterization of melanocyte samples captured from different anatomic regions (IE and HF bulge) of six NBUVB-treated vitiligo patients. Of the total 54,009 genes assessed by RNA-seq, 39 (0.07%) were differentially expressed in the melanocyte samples from NBUVB-treated vitiligo bulge compared with the patient-matched melanocyte samples from NBUVB-treated vitiligo IE, including 27 significantly up-regulated genes (0.05%) and 12 significantly down-regulated genes (0.02%) (false discovery rate-adjusted Q-value ≤ 0.05). The top 10 differentially expressed protein-coding genes in the melanocyte samples are summarized in Table 1. We selected for qRT-PCR validation the top five differentially expressed genes, among which TNC (Q = 2.0 × 10^-2), fold change [FC] = 22.7), THBS1 (Q = 2.7 × 10^-2, FC = 20.2), TM9SF3 (Q = 2.7 × 10^-2, FC = 2.5), and GJB6 (Q = 2.9 × 10^-2, FC = 27.7) were up-regulated in the melanocytes of the NBUVB-treated vitiligo bulge, whereas TYR (Q = 2.7 × 10^-2, FC = 9.1) was up-regulated in the melanocytes of the NBUVB-treated vitiligo IE. We confirmed that TNC (Padj = 7.1 × 10^-3, FC = 65.8), THBS1 (Padj = 1.7 × 10^-2, only amplified in melanocyte samples from the bulge), and GJB6 (Padj = 4.9 × 10^-2, FC = 185.3) were up-regulated in bulge melanocytes of NBUVB-treated vitiligo skin and that TYR (Padj = 2.0 × 10^-3, FC = 65.0) was up-regulated in epidermal melanocytes of NBUVB-treated vitiligo skin (Figure 2a). In the IE melanocytes, we could not detect THBS1 transcript amplification in four NBUVB-treated patients, or GJB6 transcript amplification in two of the four NBUVB-treated patients.

**qRT-PCR shows differentially expressed genes in the melanocyte samples from the bulge versus epidermis of normal untreated control skin**

We tested the expression of top genes (that were found by the RNA-seq study and were validated by qRT-PCR) in melanocytes captured from normal untreated skin of six healthy control individuals (primer sequences listed in Supplementary Table S1 online). We identified a similar expression trend with that observed in the NBUVB-treated vitiligo skin: up-regulation in bulge melanocytes of TNC (Padj = 2.4 × 10^-2, FC = 11.8), GJB6 (Padj = 1.0 × 10^-3, FC = 30.5), and THBS1 (Padj = 0.15, only amplified in 4/6 bulge samples) and up-regulation in epidermal melanocytes of TYR (Padj = 7.0 × 10^-4, FC = 13.4) (See Supplementary Figure S2 online).

Next, we tested whether the expression of TNC, GJB6, THBS1, and TYR varied in the bulge melanocytes captured from untreated vitiligo skin (n = 4), NBUVB-treated vitiligo skin (n = 6), and normal skin (n = 6). We did not find...
significant variation either among the three groups tested or among any of the paired comparisons (Figure 2b).

Pathway analysis, upstream regulators, and disease functions
To better understand the biology and functional relationship among differentially expressed genes in our RNA-seq data, we performed pathway analysis using the Ingenuity Pathway Analysis tool (Qiagen Inc., Germantown, MD; available at http://www.ingenuity.com/products/ipa) and a combined melanocyte and keratinocyte data set of 1,873 differentially expressed genes ($P < 0.05$), comparing gene expression values between samples captured from the NBUVB-treated vitiligo bulge and samples captured from the NBUVB-treated vitiligo IE. We found 15 canonical pathways...
activated and one down-regulated in the bulge compared with the IE (Table 2). The top activated pathway in the bulge (with the highest Z-score = 3.16 and the lowest P-value = 1.0 × 10^{-11}) was Integrin signaling, and its component genes that were differentially expressed (P < 0.05) are provided in Supplementary Table S2 online. The top putative upstream regulator in the bulge was CTNNB1 encoding β-catenin (Z-score = 3.61; P-value = 2.0 × 10^{-16}) (see Supplementary Table S3 online), and the top activated cellular function was cellular movement (Z-score = 3.30; P-value = 1.4 × 10^{-42}) (see Supplementary Table S4 online).

### Candidate stem cell-associated gene expression analysis

To describe the stem cell gene signature in the bulge, we examined the RNA-seq expression of 189 genes from a published stem cell differentiation panel (NanoString Technologies, Seattle, WA). The top differentially expressed genes in melanocytes from the NBUVB-treated interfollicular epidermis were FZD7 (P = 4.1 × 10^{-3}, Q-value = 0.05, FC = 46.9) and GLI1 (P = 7.7 × 10^{-3}; Q-value = 0.06; FC = 49.6) (Figure 3a). We further validated these results by qRT-PCR using new laser capture rounds of melanocytes from NBUVB-treated vitiligo patients (n = 7) (FZD7: \( P_{\text{adjusted}} = 4.0 \times 10^{-3}; \) FC = 39.5 and GLI1: \( P_{\text{adjusted}} = 9.7 \times 10^{-4}; \) only amplified in bulge samples) (Figure 3b). Next, we examined whether NBUVB modulates the expression of FZD7 and GLI1 transcripts in the bulge melanocytes isolated from NBUVB-treated vitiligo skin (n = 7), untreated vitiligo skin (n = 6), or control skin (n = 6). We found that GLI1 was significantly up-regulated in the bulge melanocytes of NBUVB-treated vitiligo skin compared with untreated vitiligo skin (\( P_{\text{adjusted}} = 2.7 \times 10^{-3}; \) FC = 9.2) but did not significantly vary in other comparisons (Figure 3c). In addition, FZD7 transcript was not significantly modulated by NBUVB after multiple testing correction (\( P_{\text{adjusted}} = 0.5; \) FC = 1.5) (Figure 3c).

Next, to validate the expression of GLI1 in the bulge melanocytes in response to NBUVB, we performed immunohistochemistry using an anti-GLI1 antibody combined with anti-DCT antibody (melanocyte specific) using skin from new NBUVB-treated vitiligo patients (n = 6) and untreated patients (n = 6). GLI1 protein was expressed in the bulge melanocytes of untreated vitiligo skin, carrying the DCT(+) phenotype, and in the bulge keratinocytes of untreated vitiligo skin, carrying the DCT(−)/GLI1(+) phenotype (Figure 3d); GLI1 was also expressed in the bulge melanocytes and keratinocytes of NBUVB-treated vitiligo skin (Figure 3e) and in the melanocytes and keratinocytes of NBUVB-treated and untreated epidermis (data not shown). We observed that GLI1(−) melanocytes, carrying the DCT(+) phenotype, and GLI1(−) keratinocytes, carrying the DCT(−)/GLI1(+) phenotype, were more numerous in the untreated bulge (Figure 3d), whereas GLI1(+) melanocytes DCT(+/-) and GLI1(+) keratinocytes DCT(−)/GLI1(+) were more numerous in the NBUVB-treated bulge (Figure 3e). Intensity analysis of microscopic images showed a stronger anti-GLI1 antibody signal in the bulge melanocytes of NBUVB-treated vitiligo skin compared with the signal in untreated vitiligo skin (P = 4.4 × 10^{-3}; FC = 1.5) (Figure 3f).

We further examined in the RNA-seq data the expression of two gene targets that directly interact with GLI1: SOX9 (encoding SOX9) (Deng et al., 2015) and CTNNB1 (encoding β-catenin) (Liao et al., 2009), together with other key components of the Wnt/β-catenin pathway (FZD8, SFRP1, WIF1, and FZD8) (see Supplementary Figure S3 online). We found that the expression values of SOX9 transcript (\( Q = 2.0 \times 10^{-7}; \) FC = 1.2) and SFRP1 transcript (\( Q = 3.0 \times 10^{-7}; \) FC = 1.8) were

### Table 1. Top genes differentially expressed in the RNA-sequencing study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Change Bulge</th>
<th>P-Value</th>
<th>Q-Value</th>
<th>Known Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNC</td>
<td>22.7</td>
<td>4.0 × 10^{-6}</td>
<td>0.020</td>
<td>Promotes β-catenin-mediated transcription in the presence of ( \text{Wnt}\text{3a} )</td>
<td>Hendou et al. 2014, Imanaka-Yoshida and Aoki, 2014</td>
</tr>
<tr>
<td>THBS1</td>
<td>20.2</td>
<td>1.4 × 10^{-3}</td>
<td>0.027</td>
<td>Downstream effector of p53, which coordinates UV-induced pigmentation; highly expressed in embryonic stem cells</td>
<td>Sundaram et al. 2011, Liu et al., 2006</td>
</tr>
<tr>
<td>TYR</td>
<td>−9.1</td>
<td>1.7 × 10^{-3}</td>
<td>0.027</td>
<td>Involved in melanin biosynthesis; highly expressed by regenerated epidermal melanocytes in the NBUVB-treated vitiligo</td>
<td>Goldstein et al., 2015</td>
</tr>
<tr>
<td>TM9SF3</td>
<td>2.5</td>
<td>2.2 × 10^{-3}</td>
<td>0.027</td>
<td>Marker of tumor invasion</td>
<td>Oo et al., 2014</td>
</tr>
<tr>
<td>GlB6</td>
<td>27.7</td>
<td>3.3 × 10^{-5}</td>
<td>0.029</td>
<td>Mutated in Clouston syndrome, which is associated with impairment of hair growth; expressed in human skin and mouse embryo</td>
<td>Lamartine et al., 2000, Fujimoto et al., 2013</td>
</tr>
<tr>
<td>SAMD5</td>
<td>72.6</td>
<td>3.7 × 10^{-3}</td>
<td>0.029</td>
<td>Stem cell gene in the mouse bulge</td>
<td>Kadaja et al. 2014</td>
</tr>
<tr>
<td>CTNND2</td>
<td>188.1</td>
<td>5.6 × 10^{-3}</td>
<td>0.029</td>
<td>Promotes disruption of adherens junction by E-cadherin, inducing cell migration</td>
<td>Zhang et al., 2009</td>
</tr>
<tr>
<td>TRPS1</td>
<td>12.4</td>
<td>5.8 × 10^{-3}</td>
<td>0.029</td>
<td>Regulates epithelial proliferation in the mouse embryo</td>
<td>Fantauzzo et al., 2012</td>
</tr>
<tr>
<td>LAMB4</td>
<td>−26.9</td>
<td>6.1 × 10^{-3}</td>
<td>0.029</td>
<td>Identified overexpressed in malignant melanoma</td>
<td>Liu et al., 2013</td>
</tr>
<tr>
<td>SOX9</td>
<td>18.9</td>
<td>6.2 × 10^{-3}</td>
<td>0.029</td>
<td>Stem cell marker detected in ~80% of malignant melanomas</td>
<td>Jo et al., 2014</td>
</tr>
</tbody>
</table>

Abbreviations: NVUVB, narrow-band UVB.

1Top 10 protein encoding genes differentially expressed in the melanocyte samples from the NBUVB-treated vitiligo bulge compared with melanocyte samples from the NBUVB-treated interfollicular epidermis. Fold changes, P-values, false discovery rate-adjusted P-values (Q-values), and gene functions are provided.
significantly up-regulated in the melanocyte samples of NBUVB-treated vitiligo bulge compared with those of NBUVB-treated vitiligo epidermis, whereas expression differences of \textit{CTNNB1}, \textit{FZD8}, and \textit{WIF1} did not surpass the false discovery rate adjustment threshold (which was $Q \leq 0.05$) (see Supplementary Figure S3).

\textbf{DISCUSSION}

In this study, we report an application (that, to our knowledge, is previously unreported) consisting of rapid immunostaining combined with F-LCM (to isolate RNA from specific cells [melanocytes], located at specific sites [bulge and epidermis]) and with RNA-seq. In a previous study, we
showed that the RNA captured from the HF bulge and epidermal melanocytes of the NBUVB-treated vitiligo and normal skin is of satisfactory quality for qRT-PCR analysis (Goldstein et al., 2016). Our current application offers a better characterization of melanocyte populations in the regenerated epidermis and the bulge of the NBUVB-treated vitiligo skin, because it uses RNA-seq technology that has deeper coverage and higher sensitivity than qRT-PCR. We found by RNA-seq analysis and confirmed by qRT-PCR that TNC, GJB6, and THBS1 were significantly up-regulated in the melanocyte samples from the NBUVB-treated vitiligo bulge, whereas TYR was significantly up-regulated in the melanocyte samples of the NBUVB-treated epidermis (Figure 2a). We observed similar expression trends in the samples of untreated normal skin (see Supplementary Figure S2). All of these data indicate that we have successfully isolated RNA from stem-like melanocytic cells in the bulge that are differentiated melanocytes that are responsible for Clouston syndrome, which is associated with hair growth impairment (Lamartine et al., 2000).

Table 2. Top canonical pathways in the RNA-sequencing study

<table>
<thead>
<tr>
<th>Ingenuity Canonical Pathways</th>
<th>P-Value</th>
<th>Z-Score</th>
<th>Down-Regulated, n (%)</th>
<th>Up-Regulated, n (%)</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integrin signaling</td>
<td>1.0 × 10^{-10}</td>
<td>3.162</td>
<td>8/201 (4)</td>
<td>37/201 (18)</td>
<td>Melanoma progression and metastasis</td>
<td>Kaphal et al., 2005</td>
</tr>
<tr>
<td>Paxillin signaling</td>
<td>2.1 × 10^{-9}</td>
<td>2.600</td>
<td>4/101 (4)</td>
<td>24/101 (24)</td>
<td>Decreased paxillin impairs adequate generation of pro-melanoma signals</td>
<td>Velasco-Velazquez et al., 2008</td>
</tr>
<tr>
<td>ILK signaling</td>
<td>3.6 × 10^{-8}</td>
<td>3.569</td>
<td>7/186 (4)</td>
<td>31/186 (17)</td>
<td>Pro-proliferative roles on several types of cancers, including melanoma</td>
<td>Dai et al., 2003</td>
</tr>
<tr>
<td>Mouse embryonic stem cell pluripotency</td>
<td>2.0 × 10^{-7}</td>
<td>2.858</td>
<td>4/95 (4)</td>
<td>20/95 (21)</td>
<td>Pro-proliferative roles</td>
<td>Niwa et al., 2007</td>
</tr>
<tr>
<td>Regulation of eIF4 and p70S6K signaling</td>
<td>2.2 × 10^{-7}</td>
<td>2.138</td>
<td>4/145 (3)</td>
<td>27/145 (19)</td>
<td>Pro-proliferative roles</td>
<td>Flynn et al., 1996</td>
</tr>
<tr>
<td>Rac signaling</td>
<td>3.2 × 10^{-7}</td>
<td>2.449</td>
<td>7/104 (7)</td>
<td>18/104 (17)</td>
<td>Controls melanocyte dendricity</td>
<td>Scott et al., 2003</td>
</tr>
<tr>
<td>Signaling by rho family GTPases</td>
<td>2.2 × 10^{-6}</td>
<td>2.667</td>
<td>10/234 (4)</td>
<td>30/234 (13)</td>
<td>Controls melanocyte dendricity</td>
<td>Scott et al., 2003</td>
</tr>
<tr>
<td>Calcium signaling</td>
<td>2.7 × 10^{-6}</td>
<td>2.132</td>
<td>7/178 (4)</td>
<td>26/178 (15)</td>
<td>Regulator of keratinocyte differentiation</td>
<td>Bilde et al., 2012</td>
</tr>
<tr>
<td>EIF2 signaling</td>
<td>5.9 × 10^{-6}</td>
<td>3.638</td>
<td>2/184 (1)</td>
<td>31/184 (17)</td>
<td>Key component of the translation initiation system in living cells</td>
<td>Stolboushkina and Garber, 2011</td>
</tr>
<tr>
<td>Role of NANOG in mammalian embryonic stem cell pluripotency</td>
<td>1.3 × 10^{-5}</td>
<td>2.646</td>
<td>6/111 (5)</td>
<td>17/111 (15)</td>
<td>NANOG functions as trigger for the reprogramming process in human cells</td>
<td>Silva et al., 2009</td>
</tr>
<tr>
<td>Melanocyte development and pigmentation signaling</td>
<td>2.0 × 10^{-5}</td>
<td>2.065</td>
<td>5/84 (6)</td>
<td>14/84 (17)</td>
<td>Melanocyte proliferation and differentiation</td>
<td>Costin and Hearing, 2007</td>
</tr>
<tr>
<td>PAK signaling</td>
<td>4.8 × 10^{-5}</td>
<td>2.357</td>
<td>3/89 (3)</td>
<td>16/89 (18)</td>
<td>Promotes ζ-MSH/UVB-induced melanogenesis</td>
<td>Yun et al., 2015</td>
</tr>
<tr>
<td>Wnt/Ca2+ pathway</td>
<td>7.8 × 10^{-5}</td>
<td>2.887</td>
<td>2/56 (4)</td>
<td>12/56 (21)</td>
<td>Putatively involved in the invasion and metastasis of melanoma</td>
<td>Yang and Qian, 2014</td>
</tr>
<tr>
<td>IGF-1 signaling</td>
<td>1.6 × 10^{-4}</td>
<td>2.496</td>
<td>5/97 (5)</td>
<td>14/97 (14)</td>
<td>Regulates keratinocyte shape and migration</td>
<td>Haase et al., 2003</td>
</tr>
<tr>
<td>FGF signaling</td>
<td>8.1 × 10^{-4}</td>
<td>2.500</td>
<td>3/85 (4)</td>
<td>13/85 (15)</td>
<td>Regulates cellular proliferation, survival, migration, and differentiation</td>
<td>Turner and Grose, 2010</td>
</tr>
<tr>
<td>RhoGDI signaling</td>
<td>1.1 × 10^{-3}</td>
<td>–2.191</td>
<td>9/173 (5)</td>
<td>22/173 (13)</td>
<td>Rho family GTPase inhibitor; suppresses melanoma cell growth</td>
<td>Wang et al., 2011</td>
</tr>
</tbody>
</table>

Abbreviation: MSH, melanocyte stimulating hormone.

1Top canonical pathways generated by combined datasets of melanocyte and keratinocyte samples from the NBUVB-treated vitiligo bulge versus NBUVB-treated vitiligo interfollicular epidermis (differentially expressed genes, P < 0.05). P-values, Z-scores, the number of up-regulated or down-regulated genes in each pathway, gene functions, and reference articles are provided.

NB Goldstein et al.
GLI1 and β-Catenin Signaling in Vitiligo Repigmentation
TYR encodes tyrosinase, the major enzyme of melanin biosynthesis, and is highly expressed in melanocytes undergoing differentiation (Cichorek et al., 2013), such as epidermal melanocytes. Using immunostaining and qRT-PCR, we previously identified TYR up-regulation in the epidermal melanocytes of NBUVB-treated vitiligo skin and unremarkable TYR expression in the bulge melanocyte precursors (Goldstein et al., 2015, 2016). Indeed, our RNA-seq
study confirmed the low expression of TYR in the NBUVB-treated vitiligo bulge melanocytes (Figure 2a), with no significant increase in response to NBUVB (Figure 2b) compared with untreated vitiligo. Although the epidermal melanocytes in this study are presumed to be fully differentiated in contrast to those in the bulge, it is likely that a subpopulation of epidermal melanocytes in treated skin could be only partially differentiated and thus share expression of some precursor genes with the bulge melanocytes. Thus, it is possible that some melanocyte stem cell markers are present in both populations. In future studies, we might compare the expression values from epidermal melanocytes in NBUVB-treated skin with values in the NBUVB-treated nonlesional vitiligo skin, assuming that these melanocytes are enriched in genes associated with regeneration process, having pro-migratory, pro-proliferative, and pro-differentiation roles. Another useful comparison is with the expression values in the epidermal melanocytes of healthy normal skin, assuming that these melanocytes are fully differentiated.

In addition, our application identified the integrin pathway as the top pathway up-regulated in the NBUVB-treated bulge melanocytes, along with 14 other significant pathways (Table 1), many of which have been previously associated with either melanoma or with melanocyte/keratinocyte proliferation, migration, differentiation, and stemness control.

We found by RNA-seq (Figure 3a) and confirmed by qRT-PCR (Figure 3b) that GLI1, a candidate stem cell-associated gene, was significantly up-regulated in the melanocyte precursors of the NBUVB-treated vitiligo bulge compared with the melanocytes of NBUVB-treated vitiligo epidermis. Also using qRT-PCR (Figure 3c) and immunostaining (Figure 3d), we identified that GLI1 was significantly modulated by NBUVB in the bulge, with higher transcript and protein expression observed in the melanocyte samples from the NBUVB-treated vitiligo bulge compared with melanocyte samples from the untreated vitiligo bulge. Our immunostaining study showed that the GLI1 was up-regulated in both melanocytes and keratinocytes of NBUVB-treated and untreated vitiligo bulge, indicating that this may be an important bulge-associated gene involved in the response to the NBUVB in the HF niche. GLI1, an effector of the Shh pathway, is required for melanocyte proliferation and for melanoma growth and metastasis (Barakat et al., 2013; Santiago-Walker and Herlyn, 2010). The Shh and Wnt/β-catenin pathways can interact through GLI1's regulation of both nuclear localization and transcriptional activity of β-catenin (Liao et al., 2009). In this study, β-catenin was identified as the top upstream regulator in the melanocyte precursors of NBUVB-treated vitiligo bulge (see Supplementary Table S3). This suggests that in vitiligo patients, NBUVB can act as an essential co-activator of CTNNB1 transcription in bulge melanocytes, which interact with GLI1 to induce melanocyte proliferation, migration, and differentiation (Figure 4a–c).

Moreover, our RNA-seq analysis shows that the increased expression of GLI1 was associated with increased expression of SOX9 (see Supplementary Figure S3) in the melanocyte samples from the NBUVB-treated vitiligo bulge compared with NBUVB-treated vitiligo epidermis. This supports our previous qRT-PCR study that identified SOX9 as a stem cell gene in the bulge of untreated normal skin and NBUVB-treated vitiligo skin (Goldstein et al., 2016). These findings suggest that GLI1 and SOX9 may interact in the melanocyte precursors of the human HF bulge to influence proliferation and/or stemness. A SOX9-GLI1 functional relationship has been previously reported: SOX9 was involved in the maintenance of GLI1 in RK3E cells, and repression of SOX9 in pancreatic ductal adenocarcinoma cells resulted in the reduction of endogenous GLI1 levels. Furthermore, mRNA analysis of Panc-1 cells transfected with SOX9-small interfering RNA showed synchronous loss of both SOX9 and GLI1 signals (Deng et al., 2015), and ectopic Sox9 expression induced the expression of Gli1 in maturing chondrocytes of chick embryos (Zeng et al., 2002).

In summary, we present a working model on the effects of NBUVB in the human vitiligo bulge (Figure 4a and b). Under NBUVB, the intercellular adhesion molecules (TNC, Gjb6, and THBS1) and FZD7 work together with GLI1, through β-catenin nuclear translocation and SOX9 constitutive expression in the bulge, to modulate the balance between stemness and activation of melanocyte precursors in the bulge of vitiligo skin. Furthermore, the GLI1 activation process is followed by melanocyte proliferation, migration, and differentiation (Figure 4b). Our previous comprehensive reviews have summarized essential signals and pathways that promote epidermal melanocyte regeneration by follicular melanocytes and that regulate the balance between stemness and differentiation states of melanocytes and keratinocytes (Birlea et al., 2017a, 2017b), including in vitro studies and studies on animal and human models of repigmentation. These genes and pathways include p53 and Wnt/β-catenin pathways; integrins, cadherins, tetraspanins, and metalloproteinases; and transforming growth factor-β (TGF-β) and its effector PAX3. Future functional studies focused on the bulge stem cell genes identified in this study (Figure 4) will examine their molecular interaction with the pathways and signals already known to be involved in the repigmentation process.

Our long-term goal is to improve the treatment outcome for vitiligo through identification of molecules that activate melanocyte precursors in the HF bulge. Because existing F-ellipse techniques were not adequate for these studies (Amoh et al., 2012; Ohyama et al., 2006; Xu et al., 2003), we developed this application (which, to our knowledge, is previously unreported) to isolate and characterize melanocyte populations in the human HF and epidermis. There are several important characteristics of this model: (i) the ability to perform a rapid immunostaining protocol that minimizes RNA degradation instead of the usual overnight antibody incubation, (ii) the ability to capture RNA from specific cells located in specific anatomic sites, and (iii) the ability to capture the cells from their natural microenvironment. Even so, there are limitations of our method (modest RNA contamination from neighbor cells, small number of cells captured, and RNA degradation), which we have tried to minimize. Contamination with RNA material of neighboring cells was minimized by using a small-diameter laser pulse (of ~16 μm). The small number of cells harvested was not a
Figure 4. Hypothetical model of the effects of NBUVB on the bulge and epidermis of human vitiligo skin during repigmentation. (a, b) Scheme of melanocyte-induced proliferation, migration, and differentiation through GLI1 activation. In human vitiligo skin, bulge-specific genes GJB6, THBS1, TNC, and FZD7 (blue shapes) are expressed in the melanocyte precursors in the bulge at similar levels (a) before and (b) after NBUVB treatment. These genes are proposed to be involved in cellular adhesion and in maintaining stemness in the bulge melanocyte precursors through β-catenin signaling. Epidermal melanocytes are absent in the interfollicular epidermis (IE) of untreated vitiligo skin, illustrated by the decreased height of all triangles, and their absence in the IE in a. In contrast, TYR (brown triangles) is expressed at low levels in both untreated and NBUVB-treated bulge melanocyte precursors and is expressed at significantly higher levels in the regenerated epidermal melanocytes of NBUVB-treated vitiligo skin, indicating active melanogenesis. GLI1 expression (red triangles) is significantly higher in the NBUVB-treated bulge melanocytes of vitiligo skin compared with untreated vitiligo skin, suggesting a role for GLI1 in the repigmentation process. We identified SOX9 constitutively expressed by the melanocyte precursors in the human bulge of normal and vitiligo skin (Goldstein et al., 2016). GLI1 also induces the bulge melanocyte and keratinocyte precursors by NBUVB is supposed to be influenced by nuclear translocation of β-catenin, and by interaction with SOX9, with a shift in the balance of maintaining stemness to greater differentiation, proliferation, and migration. (c) Cross-talk activation between β-catenin and GLI1 under NBUVB. β-catenin can enhance the transcriptional activity of GLI1 (Maeda et al., 2006), likely through regulation of the β-catenin key downstream targets IGF2BP1 (CRD-BP) and myc (Noubissi et al., 2006). Once activated by β-catenin, IGF2BP1 and myc can activate GLI1, likely by binding to the GLI1 mRNA coding region (Noubissi et al., 2009; Varnat et al., 2010). GLI1 also regulates β-catenin at the transcription level, through its targets SNAIL, WNT, and SFRP1 (Song et al., 2015). GLI1 can induce SNAIL expression, which then interacts with β-catenin and stimulates its expression activity at the transcription level (Song et al., 2015) and at the protein level (Li et al., 2007). SFRP1 and Wnt have opposite effects on β-catenin signaling, as a negative feedback or as a control mechanism after GLI1 induction, to prevent overactivation of the Wnt/β-catenin pathway. SFRP1 is a melanocyte stem gene that we identified up-regulated in the bulge of NBUVB-treated vitiligo skin and normal skin (Goldstein et al., 2016). Experimental evidence used for this figure was generated in either human models; in Gli1 transgenic mice; or on in vitro experiments performed on rat kidney cells, murine LLC-11 hepatocellular carcinoma cells, and human stomach, colon, and lung cancer cells. ##, cytoplasmic β-catenin; ⦿, nuclear β-catenin; +, baseline stimulation in the untreated vitiligo bulge; +++, increased stimulation in the NBUVB-treated vitiligo bulge; *, significant upregulation of GLI1 in the NBUVB-treated vitiligo bulge vs. untreated vitiligo bulge; MC, melanocyte; NBUVB, narrow-band UVB.
major liability in previous studies that successfully analyzed RNA after F-LCM (Dolter et al., 2001; Nakamura et al., 2007; Vandewoestyne et al., 2013). We decreased RNA degradation by optimizing the rapid immunostaining protocol, using the Illumina HiSeq 2000 sequencer, which processed only short RNA fragments (150–350 bp), and designing short amplicons (≤150 bp in our case) for qRT-PCR confirmation (Vandewoestyne et al., 2013).

Taken together, our data suggest that our F-LCM samples are enriched in target cells that are anatomically distinct and that we can successfully use RNA-seq to analyze changes of melanocyte signals associated with NBUVB-induced repigmentation in human vitiligo. The HF bulge signals and pathways activated in the melanocyte precursors presented here (Figure 4a and b) are, to our knowledge, previously unreported in the HF bulge of human vitiligo or in untreated normal skin. Most of them have been associated with cell proliferation and migration in different malignancies or in organ formation in utero, suggesting that repigmentation initiation in the bulge shares common aspects with carcinogenesis and embryonic development.

**MATERIALS AND METHODS**

**Tissue sample collection and processing for RNA studies**

Skin biopsy samples from the untreated depigmented skin of six vitiligo patients and from the repigmented skin of 10 vitiligo patients treated with NBUVB were collected in the Dermatology Clinic at University of Colorado Hospital (see Supplementary Table S5 online). The study was approved by the Human Subjects Committee at the University of Colorado, and written informed consent was obtained from all subjects. Untreated normal human skin from six control subjects was either purchased from the National Disease Resource Interchange (Philadelphia, PA) or obtained from the Skin Cancer Biorepository at the University of Colorado (Aurora, CO).

**HF bulge mapping**

To locate the HF bulge in our samples, frozen or formalin-fixed paraffin-embedded transverse sections were immunostained with a combination of anti-keratin 15 and anti-desmin antibodies every 10th slide (Goldstein et al., 2016).

**Rapid fluorescent immunostaining and F-LCM**

Frozen sections were immunostained using the NKI-beteb antibody. F-LCM was performed with an infrared laser microdissection system, under direct fluorescent microscopic visualization (Goldstein et al., 2016). From each sample, we captured 100 NKI-beteb+ melanocytes from the epidermal basal layer, 100 NKI-beteb+ cells from the bulge-outlet root sheath, and 100 adjacent keratinocytes (NKI-beteb−) from each of these two regions.

**RNA extraction, amplification, whole-transcriptome RNA-seq, and data analysis**

We analyzed the RNA isolated from melanocyte and, separately, keratinocyte samples laser captured from the repigmented skin of six vitiligo patients treated with NBUVB for 3–4 months. Total RNA extracted was amplified. Good RNA quality required an RNA integrity number of 6.5 and a concentration of greater than 500 pg/μl. Approximately more than 1 ng total RNA was used for the final libraries and was sequenced with Illumina HiSeq 2000. The RNA-seq reads from each library were aligned to the human genome (hg19). Transcripts were assembled from the aligned reads, and gene expression levels were estimated. From the global transcription, we tested the accuracy of our data by principal component analysis, which assessed the overall similarity between samples. We used a paired t test in R to analyze the genes differentially expressed in the melanocytes captured from the NBUVB-treated bulge compared with the NBUVB-treated IE. To correct for multiple testing, we used the false discovery rate with a significance threshold of Q ≤ 0.05. From the differential gene expression, we identified the significantly modulated canonical pathways, the upstream regulators, and the gene functions that were critical in the NBUVB-treated bulge compared with the NBUVB-treated IE using the Ingenuity Pathway Analysis tool.

**qRT-PCR validation of RNA-seq results**

To confirm the top genes differentially expressed in the melanocytes captured from NBUVB-treated bulge and NBUVB-treated IE, we used biopsy samples from four NBUVB-treated vitiligo patients and performed new F-LCM sessions of melanocytes, followed by total RNA extraction and amplification (as described in the Supplementary Materials and Methods online). The resulting cDNA was subjected to qRT-PCR, using primers that we designed (see Supplementary Table S1). For each sample, expression values were calculated using the standard ΔCt method normalized to ACTB expression. Paired t tests were used to compare the average ΔCt values for NBUVB-treated samples collected from IE versus bulge (P < 0.05). Differences in gene expression in the bulge melanocyte capture from normal skin, untreated vitiligo skin, and NBUVB-treated vitiligo skin were analyzed with one-way analysis of variance followed by Tukey test for multiple comparisons (P adjusted < 0.05).

**Immunohistochemistry**

Vitiligo patients’ demographics and collection methods used for immunostaining are presented in Supplementary Table S6 online.

The antibodies used for this study are listed in the Supplementary Materials and Methods. Standard procedures were used for immunohistochemistry. To quantify the GLI1 marker’s expression, we measured the average signal intensity of the anti-GLI1 antibody in melanocytes for each patient sample. The differences in signal intensity between the bulge-outlet root sheath of NBUVB-treated vitiligo skin and the bulge-outlet root sheath of untreated vitiligo skin were compared with a one-tailed unpaired t test.

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

**ACKNOWLEDGMENTS**

This work was supported by a Research Scholar Award in Vitiligo from the American Skin Association to Stanca A. Birlea and by University of Colorado School of Medicine F-LCM Shared Resource funded by the National Institutes of Health/National Center for Advancing Translational Sciences Colorado Clinical and Translational Science Awards grant UL1 TR001082. We are grateful to our team in the Dermatology Clinic at University of Colorado Hospital (UCH) for sample collection, Kathleen Ryan-Morgan and Susan Chalmers, We thank Adriaan van Bokhoven, Zachary Grasmick, and Nicole Spoelstra from the Laser Capture Microdissection Core for their valuable support with the laser capture machine. We are grateful to Manabu Ohyama, Randall Cohrs, and Mark Bungaroo for valuable advice on laser capture microdissection technique and quantitative real-time PCR. We acknowledge the National Disease Research Interchange for providing human skin samples.

**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2017.09.040.

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