Genome-wide transcriptome analysis of human epidermal melanocytes

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Abstract

Because human epidermal melanocytes (HEMs) provide critical protection against skin cancer, sunburn, and photoaging, a genome-wide perspective of gene expression in these cells is vital to understanding human skin physiology. In this study we performed high throughput sequencing of HEMs to obtain a complete data set of transcript sizes, abundances, and splicing. As expected, we found that melanocyte specific genes that function in pigmentation were among the highest expressed genes. We analyzed receptor, ion channel and transcription factor gene families to get a better understanding of the cell signalling pathways used by melanocytes. We also performed a comparative transcriptomic analysis of lightly versus darkly pigmented HEMs and found 16 genes differentially expressed in the two pigmentation phenotypes; of those, only one putative melanosomal transporter (SLC45A2) has known function in pigmentation. In addition, we found 166 genes with splice isoforms expressed exclusively in one pigmentation phenotype, 17 of which are genes involved in signal transduction. Our melanocyte transcriptome study provides a comprehensive view and may help identify novel pigmentation genes and potential pharmacological targets.

Keywords

RNA-Seq; melanocytes; transcriptome; melanosomes; pigmentation; signal transduction

Introduction

Human epidermal melanocytes (HEMs) play a critical role in protecting our skin from sunburn, photoaging, and skin cancer [1]. HEMs are located on the basal layer of the epidermis and are responsible for the synthesis of the photoprotective pigment melanin [2].

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Impaired melanocyte function can have severe consequences such as increased skin cancer risk, premature skin aging, or pigmentation disorders (i.e. vitiligo and albinism). Skin cancer is the most common form of cancer in the US and melanoma, resulting from melanocyte transformation, accounts for ~9000 deaths annually in the United States alone [3]. Thus, obtaining a better understanding of melanocyte function and human skin pigmentation is key to identifying novel targets for the treatment of skin cancer and pigmentation disorders.

Current insight into melanocyte function and human pigmentation has been based in part on comparative genomics studies using mouse coat color genes. Of the 378 loci that affect mouse coat color, 171 genes are cloned and 207 remain unidentified [4], suggesting that the molecular mechanisms that regulate pigmentation are far from being elucidated. With the recent advances in high-throughput sequencing technologies the identification of such pigmentation genes might become feasible.

The constitutive pigmentation of the skin is primarily determined by the amount of melanin produced in epidermal melanocytes and is closely correlated with the incidence of skin cancer, indicating that melanin has an important photoprotective function [5, 6]. Indeed, in the United States, the incidence of basal and squamous cell carcinomas is 50 times lower in African Americans compared to Caucasians, while melanoma rates are 13 times lower in dark compared to light skin [7-9]. Melanin is produced and stored in organelles named melanosomes using a specific set of melanocyte-specific proteins. Some of these pigmentation proteins are enzymes involved in melanin synthesis [e.g. tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1), and tyrosinase related protein 2 (DCT)], while others are structural proteins or have unknown functions. Microphthalmia transcription factor (MITF), considered the master regulator of melanocyte function, modulates the transcription of many of the pigmentation genes [10, 11]. Mutations in seven of the pigmentation genes result in ocular or oculocutaneous albinism, characterized by reduced pigment levels in the skin and eye [12].

The protective function of melanin and the identification of pigmentation proteins raised the question of what accounts for the difference between dark and light skin. It was hypothesized that the main melanogenic enzyme TYR is expressed at higher levels in melanocytes from dark skin, thus producing more melanin. However, early studies showed that the mRNA and protein levels of TYR were similar in light and dark skin, but the activity of the enzyme appeared to be different between the two skin types, being correlated with the amount of cellular melanin [13-16]. What regulates the activity of TYR is not well understood; one possibility is that it depends of the levels of TYRP1 and/or DCT, which might be higher in dark skin [17, 18]. Recent studies suggested that different pigmentation phenotypes could be the result of various combinations of single nucleotide polymorphisms (SNPs) in some pigmentation genes [19]. One particular SNP resulting in a point mutation in the melanosomal protein mutated in oculocutaneous albinism IV (OCA4 or SLC45A2) shows strong correlation with skin color [20, 21]. Moreover, the mRNA for the allele present in light skin was found to be higher than the allele present in dark skin [22]. Thus, the molecular mechanisms that determine and regulate skin color are yet to be revealed.

The advent of high-throughput RNA sequencing (RNA-Seq) has provided a more sensitive and dynamic way to study mRNA expression. RNA-Seq results contain less noise and have higher specificity compared with microarray experiments. Unlike microarray experiments, RNA-Seq provides quantitative data at single-base resolution, information on transcript size, and is not limited to the number of known genes and transcript isoforms at the time of the study [23, 24]. In addition, RNA-Seq data can be reanalyzed as sequence databases are updated. Expression profiling of human epidermal melanocytes using Affymetrix microarrays was used as a reference point for changes in melanoma lines in a study that identified only 14,500 transcripts in normal melanocytes [25]. Another microarray study sequenced melanocytes from diverse geographical origin and found highly homogenous gene expression profiles among melanocytes from skin with different pigmentation levels [18]. The recent expansion of the transcriptome to over 30,000 known protein coding transcripts [26] and the critical role of melanocytes in normal and abnormal skin physiology, make the reassessment of the melanocyte transcriptome an important task.

In this study we performed RNA-Seq on lightly and darkly pigmented human epidermal melanocytes (HEMs) using the Illumina HiSeq 2000 platform. The vast data set obtained was used to perform a comparative transcriptomic analysis of mRNA expression levels in lightly versus darkly pigmented HEMs. This analysis provides an unbiased approach to detect novel regulators of melanin synthesis, providing insight into genes critical to melanocyte function. By compiling the gene expression data into specific families of cellular signalling molecules, we bring to light potential pharmacological targets and genes important for melanocyte signal transduction. This genome-wide trancriptome analysis may serve as a valuable resource for investigating human melanocyte function and provide insight into therapeutic targets for skin cancer and pigmentation disorders.

Material and Methods

Sample collection, library preparation and sequencing

Lightly and darkly pigmented primary human epidermal melanocytes (HEMs) from neonatal foreskin (Life Technologies/Gibco) were cultured in Medium 254 and Human Melanocyte Growth Supplement (HMGS2, Life Technologies/Gibco). The four HEM lines used for this study (HEM-D1, -D2, -L1, -L2) were each derived from a different donor; all lines were propagated in culture under identical conditions for 15 population doublings. Total HEM RNA was isolated using the mirVana miRNA Isolation Kit (Ambion) and its quality was assessed using an Agilent 2100 Bioanalyzer: RNA Integrity Number (RIN) 8.7 for all samples, in agreement with Illumina recommended RIN 8. cDNA libraries were prepared with 4μg total RNA using standard Illumina protocols (TruSeq RNA Sample Preparation Kit) and resulted in cDNA fragments with 229 bp average size, or 355bp including adapter sequences. Each cDNA library was sequenced with 50 bp single-read chemistry using the Illumina HiSeq 2000 system. All of the sequence files have been submitted to the GenBank Sequence Read Archive for public access [Accession No. **SRP039354 http://dx.doi.org/ 10.7301/Z0MW2F2N**].

Data analysis and annotation

The computational pipeline used for data analysis is shown in **Supplemental Figure S1**. The quality of the raw sequencing data was checked using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the quality scores of the reads for each library and for each position in the read were above 30, which is defined as high quality (**Supplemental Figure S2**). The sequencing data in FASTQ format was then mapped against NCBI build 37.2 of the human genome using Bowtie 2 (version 2.1.0.0) [27]. RNA sequencing metrics were obtained using Picard tools (http://picard.sourceforge.net/) (**Table 1**). Spliced junctions were identified using Tophat (version 2.0.8) [28] and transcript abundance estimates were performed using Cufflinks (version 2.1.1) [29]. EdgeR was used to perform differential gene expression analysis between samples with different pigmentation levels [30, 31]. Genes differentially expressed were considered significant if they had a FDR adjusted p-value < 0.05 [32]. For the isoform analysis we imported the Cufflinks isoform expression data of lightly and darkly pigmented HEMs into Microsoft Excel. Using Excel functions we identified the isoforms only present in HEM-L or HEM-D and excluded those with high degree of variability (standard error mean > 35% the average FPKM).

qPCR analysis

3μg of total RNA was extracted from the same HEM lines used for RNAseq using the RNeasy Plus Kit (Qiagen) and reverse transcribed (RT) using SuperScript III kit (Life Technologies). The resulting cDNA was used for qPCR validation of each biological replicate analysed by RNA-Seq. Reactions were prepared according to the manufacturer's protocol using SYBR Select Master Mix (Invitrogen) and cycled on a VIIA-7 Real-Time PCR System (Applied Biosystems). β-actin was used as an internal control and all reactions were run in triplicate. mRNA levels were quantified by calculating average $2^{-\text{Ct}}$ values, where Ct is the cycle number for the control and target transcript at the chosen threshold. Ct = $\text{Ct}_{\text{target}}$ - $\text{Ct}_{\beta\text{-actin}}$ was calculated by subtracting the average Ct of β-actin from the average Ct of the target transcript. Primers were designed to span an exon-exon junction to avoid amplification of any contaminate DNA. Primers used for SLC45A2, OCA2, and SLC24A5 qPCR were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) and were as follows: SLC45A2 (NM_016180.3) - F: CCCTGTACACTGTGCCCTTT and R: CTTCCCTCTCACGCTGTTGT, OCA2 (NM_000275.2) - F: GTGTGCAGGGATTGCAGAAC and R: ACATCCCAACAGTGCAGGAC, SLC24A5 (NM_205850.2) - F: GCAGCAGGAACAAGCATACC and R: ATGGAATACCAAGGCACAACA.

The relative difference in expression between the two pigmentation phenotypes (HEM-D and HEM-L) was calculated as fold change in HEM-L vs. HEM-D (HEM-L/HEM-D); for RNA-Seq it was calculated by dividing average FPKM of HEM-L by that of HEM-D and for qPCR by dividing the average $2^{-\text{Ct}}$ of HEM-L by that of HEM-D.

Melanin quantification

HEMs were grown to 70 – 90% confluence in 35 mm Petri dishes and melanin collected and quantified as previously described [33]. Briefly, after cell lysis soluble and insoluble

fractions were separated by centrifugation. The soluble fraction was used to determine the total amount of protein in each sample using a Bradford assays (Bio-Rad Laboratories). The insoluble fraction containing melanin was resuspended in 100 μl of 1 M NaOH and incubated at 85°C until melanin was fully dissolved (15 – 30 min). Melanin was quantified by comparing the optical density of samples at 405 nm with a standard curve generated with serial dilutions of synthetic melanin (Sigma) dissolved in 1 M NaOH. Average cellular melanin values were calculated as the ratio between total melanin and total protein from the same dish of cells. All melanin quantifications were performed in triplicate to account for dish-to-dish variability.

Statistical Analysis

P-values were calculated using an unpaired two-tailed Student's t-test with $P < 0.05$ considered significant. For the differential gene expression analysis a FDR adjusted p-value < 0.05 was considered significant [32]. qPCR and melanin quantification were performed using the same biological replicates used for RNA-Seq analysis.

Results and Discussion

RNA sequencing of human epidermal melanocytes

Because melanocytes in the human epidermis are important for the constitutive pigmentation of our skin, which correlates with the risk of skin cancer and is also affected by many pigmentation disorders, we sought to obtain a global gene expression profile of melanocytes from skin with different pigmentation levels. We used primary human epidermal melanocytes (HEMs) in culture to perform a genome-wide transcriptome analysis. We used samples derived from lightly pigmented (HEM-L) and from darkly pigmented melanocytes (HEM-D), which have ~ 7-fold higher cellular melanin content than the lightly pigmented ones (**Fig. 1A**). To ensure biological replication, we used RNA sequencing (RNA-Seq) on four different libraries: two libraries derived from individual donors of darkly pigmented melanocytes (HEM-D1 & HEM-D2) and two from individual donors of lightly pigmented melanocytes (HEM-L1 & HEM-L2). Each HEM cDNA library was derived by isolation of polyA(+) mRNA and reverse transcription with random hexamer primers, and sequenced using 50bp single-read chemistry. The total number of reads for each cDNA library varied between 78 million and 188 million for the four samples (**Table 1**). The sequenced fragments were aligned to the NCBI build 37.2 of the human genome using Bowtie 2 [27], splice junctions were detected using Tophat [28], and transcript abundances were calculated in **F**ragments **P**er **K**ilobase of exon per **M**illion fragments mapped (**FPKM**) using Cufflinks [29] (**Supplemental Figure S1**). Because FPKM reflects the expression level of the genes to which the fragments correspond, we chose a significance threshold of FPKM 0.1 to prevent analysis of transcripts with expression levels close to zero (bottom 25th percentile of transcripts had FPKM values < 0.05). Using this criterion we detected between 14,782 and 15,252 genes for all four libraries (**Table 1**). We also identified over 21,000 transcript variants across all four libraries. The gene expression statistics for each HEM cDNA library are shown in **Table 1**, while a complete data set of transcript abundances, sizes, and alternative splicing for all four libraries is found in the on-line **Supplemental Table S1**.

The expression of different genes, as measured by FPKM, was in some cases variable between the four libraries. Genes that were expressed at different levels in the two dark HEM libraries compared to the two light ones were further analysed and discussed in **Tables 5-7**. For the rest of the genes that show variable expression levels, however, variability was not linked to the pigmentation phenotype of the cells and might represent biological variation between different individuals. Because we cannot exclude technical variability during library preparation, we used the average FPKM and the standard error mean for each gene to calculate the % variability among the four different samples. We generated a set of "high confidence" genes that had less than 35% variability among all biological replicates (included in **Supplemental Table S1**).

Genes highly expressed in human epidermal melanocytes

HEMs, due to their unique ability to produce melanin, require a constant turnover of melanosomes, specialized intracellular organelles in which melanin is synthesized, stored, and transferred to neighbouring keratinocytes. The abundant presence of these unique organelles in melanocytes suggests that in HEMs highly expressed genes also encode melanosomal components such as melanogenic enzymes, structural proteins, or regulators of the melanosomal environment and function. We determined the highest expressing genes in HEMs by arranging our results in the decreasing order of their average FPKM (**Table 2**). Not unexpectedly, we found that many melanocyte specific pigmentation genes (bolded in **Table 2**) are expressed at or above the level of the common housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (**Table 2**; highlighted in dark grey background). In particular, six melanocyte specific pigmentation genes: tyrosinase (TYR), tyrosinase related protein 1 (TYRP1), tyrosinase related protein 2 (DCT), premelanosomal protein (PMEL17), glycoprotein NMB (GPNMB), and melanoma antigen 1 (MLANA) are among the 30 highest expressing genes in HEMs (highlighted in **Table 2**). PMEL17, a structural melanosomal protein important in early stage melanosome biogenesis, had the highest average expression in HEMs (FPKM = 8302.8 ± 2306.6) compared with all genes, almost an order of magnitude higher than β-actin (FPKM = 852.5 ± 140.8). TYR, TYRP1 and DCT are key enzymes involved in the melanin synthesis pathway; mutations in TYR and TYRP1 cause severe hypopigmentation and oculocutaneous albinism type 1 (OCA1) and type 3 (OCA3), respectively [34-36]. Widely expressed genes such as vimentin (VIM), ferritin heavy polypeptide (FTH1), ferritin light polypeptide (FTL), and prosaposin (PSAP) are also among the highest expressed genes.

Receptors and ion channels highly expressed in human epidermal melanocytes

Receptors and ion channels play a crucial role in cellular signalling and are often used as drug targets. To identify receptors and ion channels highly expressed in HEMs and presumably important for their function, we compiled gene family lists of all known G protein-coupled receptors (GPCRs), receptor kinases, and ion channels using the IUPHAR database and the HUGO genes repository [37], then sorted these categories in the order of their average FPKM (**Tables 3, 4, and Supplemental Table S5**). As expected, we found that several genes known to be important for melanocyte function (shown in bold) among the highly expressed receptors and ion channels.

We first sorted GPCRs and receptor kinases to find the highest expressed signalling receptor genes in HEMs. Both the endothelin receptor type B (EDNRB) and the melanocortin 1 receptor (MC1R) are among the top 25 highest expressing GPCRs in HEMs (**Table 3**). EDNRB plays an important role in melanocyte development and migration [38], while MC1R is critical for hair and skin pigmentation and plays a critical role in the UVB-induced pigmentation response [39-41]. The melanosomal receptor GPR143 (mutated in ocular albinism type 1, OA1), a receptor important for melanosome structure and biogenesis, was the highest expressing GPCR gene [42-44]. Interestingly, eleven of the top 25 GPCRs highly expressed in HEMs are orphan receptors (**Table 3**; **s**hown in dark grey background). This finding is particularly exciting because identifying endogenous ligands for these receptors and associated signalling mechanisms might uncover novel pathways important for melanocyte function. Interestingly, the serotonin receptor, 5-hydroxytryptamine receptor 2B (HTR2B), and the predicted extraocular photoreceptor encephalopsin (OPN3) are also highly expressed, yet their physiological functions in melanocytes are not known. Critical melanocyte receptor kinases such as stem cell growth factor receptor c-Kit (KIT), tyrosine-protein kinase receptor TYRO3 (TYRO3), and the hepatocyte growth factor receptor (MET) are among the 10 highest expressed receptor kinase genes **Supplemental Table S5** [45-47].

We next sorted the ion channels according to expression values in HEMs and found that among the top 25 highly expressed ion channel genes in HEMs are TRPM1, TRPM7, and TRPML3 (shown in bold in **Table 4**), known to function in regulating melanin content and pigmentation of human, mouse, and zebra fish [33, 48-50]. Interestingly, nine out of the top 25 highly expressed ion channel genes are chloride channels (**Table 4**; shown in dark grey background), suggesting that chloride regulation is important for melanocyte function. Among the highly expressed channels are the calcium activated chloride channel BEST1 [51], the intracellular CLIC1 channel [52], and the non-selective cation channel TRPV2 [53, 54]. Interestingly, TRPV2 ion channels were also found by a proteomics study in purified melanosomes [55]. In addition, the highly expressed chloride channel, CLCN7, and the two-pore channel, TPCN2, are associated with variations in pigmentation [56, 57], suggesting a role in melanosomal physiology and melanin content of melanocytes. The Transient Receptor Potential A1 (TRPA1) channel that was shown to be expressed in melanocytes and important for melanocyte responses to ultraviolet A radiation [58] is not among the highly expressed ion channels (TRPA1, FKPM = 0.3 ± 0.1), in agreement with the small size of the TRPA1 currents measured in HEMs by patch clamp [58]. This suggests that while high expression of ion channels could correlate with important physiological function, low expressing signalling proteins can also be critical for cell function. A complete list of GPCR, receptor kinase and ion channel gene expression in HEMs is found in **Supplemental Table S2**.

Transcription factor gene expression in human epidermal melanocytes

Transcription factors regulate a multitude of cellular processes by controlling the rates at which genes are transcribed. Using a previously compiled list of 1391 human transcription factor genes [59], we generated a table of the top 25 highest expressing transcription factor genes in HEMs (**Supplemental Table S6**). We found transcription factor genes important for melanocyte function such as microphthalmia-associated transcription factor (MITF), sex

determining region Y-box 10 (SOX10), and Y box binding protein 1 (YBX1) to be among the top 25 highly expressed transcription factor genes. MITF is responsible for regulating a multitude of genes necessary for melanocyte development and differentiation [60]; SOX10 is a regulator of MITF and has a major role in melanocyte development [61, 62], while YBX1 is an activator of MIA (melanoma inhibitory activity) and plays an important part in the progression of malignant melanoma [63]. A complete list of transcription factor gene expression in HEMs is found in **Supplemental Table S3**.

Differentially expressed genes and isoforms in lightly versus darkly pigmented melanocytes

HEMs contain different amounts of melanin correlated with the pigmentation phenotype of the donor. Because melanin is photoprotective, we sought to identify differentially expressed genes and isoforms in lightly vs. darkly pigmented HEMs (HEM-L vs. HEM-D). We performed a global differential gene expression analysis using edgeR software [30, 31] and found 94 genes expressed at significantly different levels (false discover rate (FDR) adjusted p-value < 0.05), with 16 genes remaining after filtering genes expressed below threshold (FPKM 0.1) [32] (**Table 5**). Apolipoprotein C-II (APOC2) had the highest fold change in expression between HEM-L and HEM-D. Interestingly, solute carrier family 45, member 2 (SLC45A2) was the only known pigmentation gene that was significantly different between the two pigmentation phenotypes. Moreover, SLC45A2 was increased 8-fold in HEM-L compared to HEM-D (shown in bold in **Table 5**), as previous studies have suggested [22].

To confirm the EdgeR results and to further compare pigmentation gene expression in melanocytes, we analysed the average FPKM values of 11 melanocyte specific pigmentation genes (as defined in [64]), as a function of pigmentation levels and represented them in groups according to their predicted function (**Table 6**). As expected, all of the pigmentation genes were expressed at high levels, some of these transcripts had high variability between samples, but, with the exception of SLC45A2, we did not detect any pigmentation gene that was significantly different in darkly vs. lightly pigmented cells (**Table 6**). The similar mRNA expression levels of the rate limiting enzyme, tyrosinase (TYR) in HEM-L and HEM-D was confirmed with average FPKM $\pm$ SEM values of 1429.8 ± 18.0 (HEM-L) and 1604.6 ± 98.9 (HEM-D), respectively [14].

SLC45A2 is predicted to function as a melanosomal transporter [65, 66], similar to P-protein and SLC24A5, but the role of these proteins in melanosomal function is not known. Moreover, mutations in all of these predicted transporters result in reduced melanin levels and oculocutaneous albinism: mutations in SLC45A2 result in oculocutaneous albinism type 4 (OCA4) [65], mutations in P-protein results in OCA2 [67], and mutations in SLC24A5 result in OCA6 [12]. To further explore differences in the expression levels of the melanosomal transporters between lightly and darkly pigmented melanocytes we represented their average FKPM as a function of pigmentation phenotype (**Fig. 1B**).

To validate the RNA-Seq data and to verify the identified difference between SLC45A2 mRNA expression in HEM-L vs. HEM-D, we measured by qPCR the mRNA levels of the three predicted melanosomal transporters: P-protein, SLC45A2, and SLC24A5 in cells derived from the same biological samples as used for RNA-Seq and corresponding to the

two pigmentation phenotypes. We then calculated for each gene the average mRNA fold increase in HEM-L compared to HEM-D for RNA-Seq and qPCR (**Fig. 1B**). In our analysis genes enriched in HEM-L would have values greater than one, while genes expressed at higher levels in HEM-D would have values less than one. Both RNA-Seq and qPCR results showed similar fold changes and confirmed our previous observations that SLC45A2 (OCA4) is expressed at significantly higher levels in HEM-L. P-protein (OCA2) was found to be higher in HEM-D by qPCR (consistent with previous studies [22, 68]) as well as SLC24A5 (OCA6), but the difference was not statistically different by RNA-Seq. Interestingly, the SLC45A2 mRNA found in the HEM-L libraries was exclusively accounted for by the 374F allele, while HEM-D libraries contained the 374L allele, in agreement with previous findings [20-22].

In addition to our EdgeR analysis of differential gene expression, we investigated whether different isoforms of the same genes were differentially expressed for each pigmentation phenotype. Using Cufflinks isoform expression data, we isolated transcripts only expressed in HEM-L or HEM-D. After filtering out transcripts with a standard error mean greater than 35% of the average FPKM, we identified a total of 103 isoforms expressed only in HEM-L and 63 isoforms only HEM-D (**Fig. 1C, <u>Supplemental Table S4</u>**). From these isoforms, we isolated the genes likely to have a signalling role and to function as potential melanogensis regulators, shown in **Table 7**. Only four signalling isoforms were identified in HEM-D (**Table 7A**) and 13 isoforms in HEM-L (**Table 7B**). Three of the differentially expressed transcripts were G protein coupled receptors (GPCR), one was a receptor kinase (RK), one was a regulator of G protein signalling (RGS3) and 12 isoforms represented transcription factors. Among those, some isoforms encoded the canonical sequence of the gene, while others were splice variants resulting in truncated proteins.

Thus, our differential analysis results, despite the limited number of biological replicates, offered valuable insight into the expression of signalling proteins in melanocytes and revealed a single melanocyte specific gene and several genes and isoforms with unknown function in pigmentation being differentially expressed as a function of pigmentation phenotype.

Conclusions

In this study, we performed global gene expression analysis of HEMs with a focus on gene families related to signal transduction. To our knowledge this study is the first report of a comparative transcriptomic analysis of lightly versus darkly pigmented HEMs using high throughput sequencing technology. This work revealed a novel set of 15 genes and 166 transcript isoforms that are differentially expressed in lightly versus darkly pigmented HEMs that are potentially related to differences in skin pigmentation. SLC45A2 was the only melanocyte specific pigmentation gene differentially expressed, suggesting that SLC45A2 may be a key regulator of melanogenesis. These results provide a comprehensive view of the HEM transcriptome and a foundation for discovering novel pharmacological targets for treatment of pigmentation disorders and melanoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

>HEMs	human epidermal melanocytes
FPKM	Fragments Per Kilobase of exon per Million fragments mapped

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Research Highlights

- The transcriptome of human melanocytes was determined using RNA sequencing.

- We compared melanocytes with different pigmentation levels from different skin types.

- Our analysis focused on cell signaling genes like receptors and ion channels.

- Pigmentation genes specific to melanocytes were expressed at very high levels.

- Our study found 15 novel genes differentially expressed in light vs. dark human melanocytes.
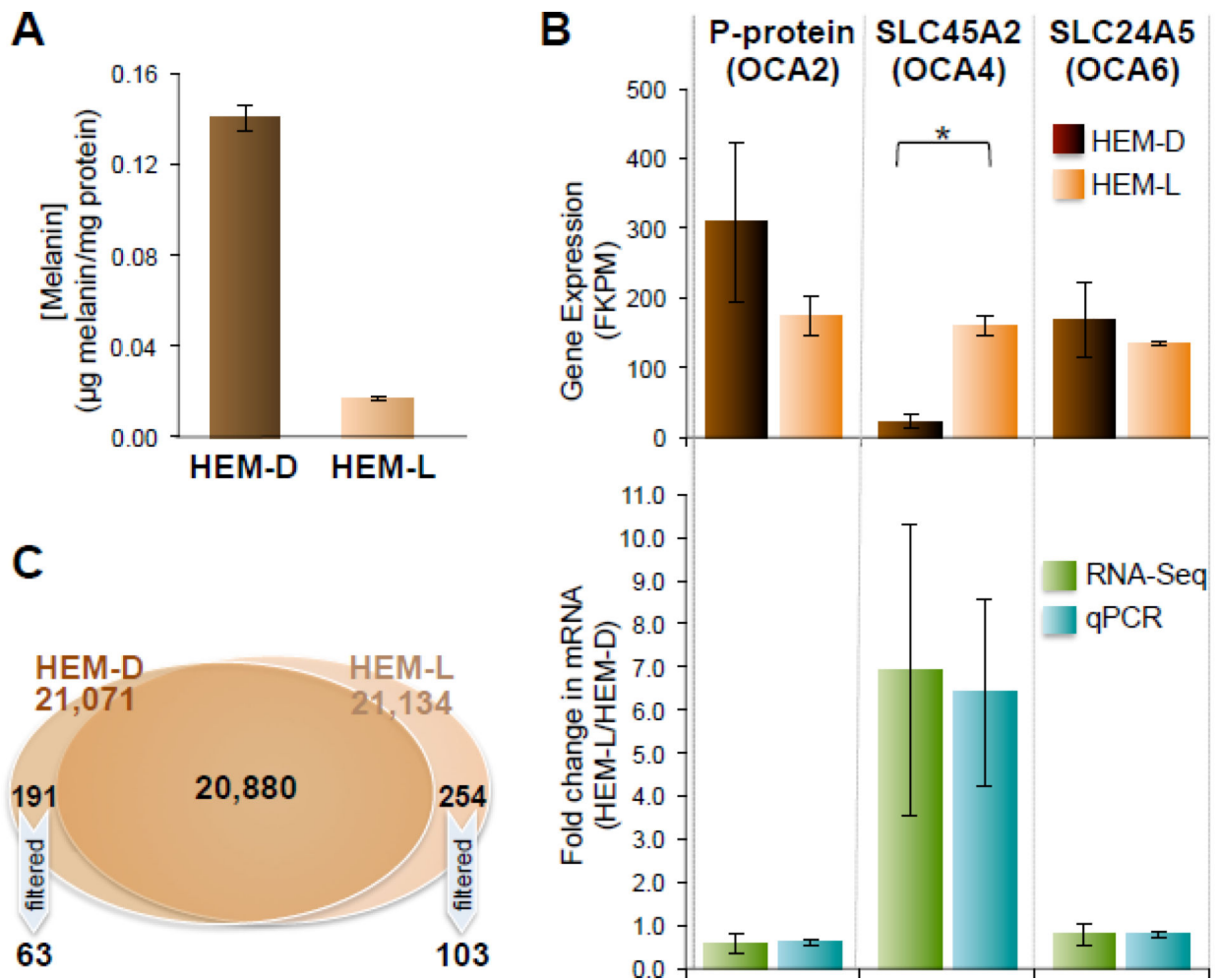
Figure 1. Differential gene and isoform expression in HEM-D vs. HEM-L

A. Average melanin concentration of HEM-D vs. HEM-L. The average melanin concentration in the two darkly pigmented cell samples (HEM-D1 & HEM-D2) was ~ 7 fold higher than the average of the two lightly pigmented cell samples (HEM-L1 & HEM-L2) (n = 4, p < 0.002). Bars represent average ± SEM.

B. Expression of predicted melanosomal transporters in HEM-L vs. HEM-D. (*top*) Average transcript abundance of P-protein (OCA2), SLC45A2 (OCA4) and SLC24A5 (OCA6) in HEM-D and HEM-L determined by RNA-Seq (n = 2). SLC45A2 is expressed at significantly higher levels in HEM-L, compared to HEM-D (p < 0.015). Bars represent average FPKM ± SEM. (*bottom*) Comparison of fold change in transcript abundance of predicted melanosomal transporters in HEM-L vs. HEM-D, as determined by RNA-Seq or qPCR. SLC45A2 is expressed at higher levels in HEM-L by both RNA-Seq and qPCR, while P-protein and SLC24A5, with values less than one, have higher expression levels in HEM-D as measured by qPCR (n = 3, p < 0.01) but are not significantly different by RNA-Seq (n = 2, p > 0.36). The error bars represent the error propagation of the standard error mean from the average $2^{-\Delta Ct}$ with respect to β-actin for qPCR, and ± the standard error mean from the average FPKM for RNA-Seq.

C. Distribution of the total number of transcripts identified from HEM-D and HEM-L. Venn diagram representing the distribution of the total number of transcripts between libraries obtained from darkly (HEM-D) and lightly (HEM-L) pigmented cells. The transcripts with FPKM < 0.1 in any of the libraries and variability >35% between the two libraries derived from the same pigmentation phenotype were filtered out.

Table 1

RNA sequencing metrics and summary analysis of gene expression in HEMs

RNA sequencing metrics of human epidermal melanocytes (HEMs) for two darkly pigmented (HEM-D1 & HEM-D2) and two lightly pigmented (HEM-L1 & HEM-L2) samples.

Library	Produced/Aligned Reads (million)	Sequenced bases (Mb)	mRNA bases (%)	Number of genes detected	Gene expression (FPKM)	
					Range	Mean
HEM-D1	78.7 / 76.4	3939.2	83.3	14,782	0.1-14645.4	27.02
HEM-D2	181.0 / 177.1	9053.1	85.3	15,252	0.1- 8627.7	31.51
HEM-L1	174.0 / 168.4	8700.0	80.0	15,060	0.1- 9180.9	28.63
HEM-L2	188.1 / 183.8	9405.2	83.9	15,071	0.1- 5347.3	24.07

The mean and median FPKM, and the FPKM range was calculated for each HEM cDNA library, as well as the number of genes detected. A threshold of FPKM   0.1 was used for all calculations.

Table 2

Highly expressed genes in HEMs

Gene Name (Gene ID)	Average FPKM ± SEM
premelanosome protein (PMEL17)	**8302.8 ± 2306.6**
tyrosinase-related protein 1 (TYRP1)	**7481.6 ± 882.4**
eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)	3172.8 ± 734.0
vimentin (VIM)	2912.1 ± 526.4
ferritin, heavy polypeptide (FTH1)	2536.3 ± 416.5
CD63 molecule (CD63)	2355.4 ± 229.4
ferritin, light polypeptide (FTL)	1848.3 ± 268.5
glycoprotein (transmembrane) nmb (GPNMB)	**1795.5 ± 447.3**
tumor protein, translationally-controlled 1(TPT1)	1722.7 ± 362.5
Prosaposin (PSAP)	1682.8 ± 327.1
ATPase, Na+/K+ transporting, alpha 1 polypeptide (ATP1A1)	1628.1 ± 255.3
tyrosinase-related protein 2 (TYRP2)	**1566.2 ± 538.9**
actin, gamma 1 (ACTG1)	1562.1 ± 86.3
melanoma antigen 1 (MLANA)	**1550.1 ± 244.1**
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	**1524.2 ± 141.1**
tyrosinase (TYR)	**1517.2 ± 65.0**
thymosin beta 10 (TMSB10)	1367.6 ± 158.3
eukaryotic translation elongation factor 1 gamma (EEF1G)	1155.6 ± 157.7
annexin A5 (ANXA5)	1139.9 ± 93.8
lectin, galactoside-binding, soluble, 3 (LGALS3)	1087.2 ± 76.5
spermidine/spermine N1-acetyltransferase 1 (SAT1)	1033.1 ± 387.8
lectin, galactoside-binding, soluble, 1 (LGALS1)	1030.8 ± 63.7
ubiquitin B (UBB)	961.3 ± 161.7
heat shock 70kDa protein 8 (HSPA8)	944.6 ± 81.0
cofilin 1 (non-muscle) (CFL1)	922.6 ± 89.3
ATP1A1 opposite strand (ATP1A1OS)	853.4 ± 134.1
actin, beta (ACTB)	**852.5 ± 140.8**
stearoyl-CoA desaturase (delta-9-desaturase) (SCD)	848.4 ± 188.4
TIMP metallopeptidase inhibitor 2 (TIMP2)	835.1 ± 143.4
G protein, beta polypeptide 2-like 1 (GNB2L1)	813.6 ± 110.9

Highly expressed genes in melanocytes were obtained by averaging their FPKM from each library. For concision, ribosomal and non-coding genes we excluded from this table (their expression values can be found in **Supplementary Table S1**). Melanocyte specific pigmentation genes are highlighted in bold and, for comparison of expression levels, commonly used housekeeping genes (GAPDH and β-actin) are shown in dark grey background.

Table 3

Top 25 highly expressed G protein-coupled receptors in HEMs

Gene Name (Gene ID)	Average FPKM ± SEM
G protein-coupled receptor 143 (OA1)	**254.7 ± 44.0**
endothelin receptor type B (EDNRB)	**211.6 ± 43.0**
G protein-coupled receptor 56 (GPR56)	**148.8 ± 29.3**
G protein-coupled receptor 137B (GPR137B)	**117.4 ± 7.1**
5-hydroxytryptamine (serotonin) receptor 2B (HTR2B)	66.4 ± 37.5
cysteinyl leukotriene receptor 2 (CYSLTR2)	29.5 ± 14.4
G protein-coupled receptor 107 (GPR107)	**25.4 ± 2.6**
coagulation factor II (thrombin) receptor (F2R)	24.6 ± 9.2
G protein-coupled receptor 108 (GPR108)	**21.5 ± 3.6**
G protein-coupled receptor 125 (GPR125)	**21.2 ± 1.5**
G protein-coupled receptor 175 (GPR175)	**14.6 ± 3.3**
G protein-coupled receptor, fam. C, group 5, member A (GPRC5A)	**13.3 ± 1.9**
melanocortin 1 receptor (MC1R)	**12.8 ± 4.9**
cadherin, EGF LAG seven-pass G-type receptor 2 (CELSR2)	11.9 ± 3.0
G protein-coupled receptor 124 (GPR124)	**11.3 ± 1.9**
CD97 molecule (CD97)	10.9 ± 1.8
encephalopsin (OPN3)	10.1 ± 1.5
G protein-coupled receptor, fam. C, group 5, member B (GPRC5B)	**9.5 ± 3.8**
smoothened, frizzled family receptor (SMO)	8.8 ± 1.3
lysophosphatidic acid receptor 6 (LPAR6)	8.5 ± 3.9
frizzled family receptor 6 (FZD6)	8.5 ± 3.1
G protein-coupled receptor 137 (GPR137)	**8.2 ± 1.3**
gonadotropin-releasing hormone (type 2) receptor 2 (GNRHR2)	7.9 ± 0.7
gamma-aminobutyric acid (GABA) B receptor, 1 (GABBR1)	7.9 ± 1.4
G protein-coupled receptor 155 (GPR155)	**7.8 ± 1.4**

The highest expressed GPCRs in melanocytes were obtained by averaging their FPKM from each library. The mean expression levels of orphan GPCRs shown in dark grey background and receptors known to be important for melanocyte function (GPR143, EDNRB, and MC1R) are in bold.

Table 4

Top 25 highly expressed ion channel genes in HEMs.

Gene Name (Gene ID)	Average FPKM ± SEM
chloride intracellular channel 1 (CLIC1)	**242.8 ± 28.6**
purinergic receptor P2X, ligand-gated ion channel, 7 (P2RX7)	78.4 ± 16.7
TRP cation channel, subfamily V, member 2 (TRPV2)	75.6 ± 16.2
chloride channel, voltage-sensitive 3 (CLCN3)	**72.4 ± 13.5**
chloride intracellular channel 4 (CLIC4)	**71.4 ± 16.7**
TRP cation channel, subfamily M, member 1 (TRPM1)	**57.8 ± 9.4**
two pore segment channel 2 (TPCN2)	52.1 ± 7.2
chloride channel, voltage-sensitive 7 (CLCN7)	**50.6 ± 8.0**
polycystic kidney disease 2 (PKD2)	31.6 ± 7.6
bestrophin 1 (BEST1)	**31.3 ± 7.4**
pannexin 1 (PANX1)	25.2 ± 2.7
anoctamin 10 (ANO10)	**23.5 ± 3.1**
anoctamin 6 (ANO6)	**21.7 ± 4.0**
potassium channel, subfamily J, member 13 (KCNJ13)	17.6 ± 9.4
purinergic receptor P2X, ligand-gated ion channel, 4 (P2RX4)	17.1 ± 1.7
mucolipin 1 (MCOLN1, TRPML1)	16.8 ± 3.3
mucolipin 3 (MCOLN3, TRPML3)	**15.5 ± 5.6**
inositol 1,4,5-trisphosphate receptor, type 3 (ITPR3)	12.6 ± 3.0
chloride channel, voltage-sensitive 6 (CLCN6)	**11.8 ± 2.3**
potassium channel, delayed-rectifier, subfamily S, member 3 (KCNS3)	10.7 ± 2.3
TRP cation channel, subfamily M, member 7 (TRPM7)	**10.2 ± 3.1**
gap junction protein, gamma 1, 45kDa (GJC1)	9.5 ± 2.7
two pore segment channel 1 (TPCN1)	8.8 ± 2.3
chloride channel, voltage-sensitive 5 (CLCN5)	**5.1 ± 0.6**
gap junction protein, alpha 3, 46kDa (GJA3)	4.8 ± 0.3

The highest expressed ion channels in melanocytes were obtained by averaging their FPKM from each library. Nine out of the 25 genes are chloride channels (shown in dark grey background). Ion channels reported to be important for melanocyte function are in bold.

Table 5

Differentially expressed genes in lightly (HEM-L) vs. darkly (HEM-D) pigmented melanocytes.

Gene Name (Gene ID)	Relative expression in HEM-L vs. HEM-D	Fold Change	FDR p-value
apolipoprotein C-I (APOC1)	Up-regulated	13	3.81×10^{-3}
apolipoprotein C-II (APOC2)	Up-regulated	117	2.74×10^{-7}
family with sequence similarity 171, member A1 (FAM171A1)	Up-regulated	12	1.81×10^{-2}
glyceraldehyde-3-phosphate dehydrogenase (GAPDHS)	Up-regulated	8	1.63×10^{-2}
leucine rich repeat containing 61 (LRRC61)	Up-regulated	20	2.23×10^{-2}
leucine rich repeat neuronal 1 (LRRN1)	Up-regulated	47	1.12×10^{-2}
protein tyrosine phosphatase N, polypeptide 2 (PTPRN2)	Up-regulated	24	3.23×10^{-2}
sarcoglycan, delta (SGCD)	Up-regulated	15	2.74×10^{-2}
solute carrier family 45, member 2 (SLC45A2)	**Up-regulated**	**8**	**4.22×10^{-2}**
tudor domain containing 12 (TDRD12)	Up-regulated	27	2.69×10^{-3}
zinc finger protein 423 (ZNF423)	Up-regulated	17	2.71×10^{-2}
claudin 1 (CLDN1)	Down-regulated	10	1.43×10^{-2}
CXXC finger protein 4 (CXXC4)	Down-regulated	19	1.59×10^{-2}
kinase insert domain receptor (KDR)	Down-regulated	8	2.21×10^{-2}
myosin VIIB (MYO7B)	Down-regulated	25	3.81×10^{-2}
protein tyrosine phosphatase, receptor type, O (PTPRO)	Down-regulated	18	4.88×10^{-2}

The number of reads that aligned to the identified genes in the HEM-L and HEM-D libraries were analyzed with the edgeR software to detect significant differences (FDR p-value < 0.05). The only pigmentation-associated gene differentially regulated (SLC45A2) is shown in bold.

Table 6

Expression of melanocyte specific pigmentation genes in lightly (HEM-L) vs. darkly (HEM-D) pigmented melanocytes.

Gene Name (Gene ID)	Function (predicted)	Average HEM-L FPKM ± SEM	Average HEM-D FPKM ± SEM
P protein (OCA2)		174.5 ± 28.7	308.3 ± 114.7
solute carrier family 45 member 2 (SLC45A2)	melanosomal transporter	**160.6 ± 14.0**	**23.2 ± 8.8**
solute carrier family 24 member 5 (SLC24A5)		134.3 ± 3.4	167.6 ± 53.2
premelanosome protein (PMEL)		7047.6 ± 1700.3	9558.0 ± 5087.4
melan-A (MLANA)	melanosome structure	1677.3 ± 415.4	1423.0 ± 390.7
G protein-coupled receptor 143 (OA1)		238.9 ± 71.2	270.5 ± 77.9
glycoprotein (transmembrane) nmb (GPNMB)	melanosomal component	1582.8 ± 302.6	2008.2 ± 1009.1
tyrosinase (TYR)		1429.8 ± 18.0	1604.6 ± 98.9
tyrosinase-related protein 1 (TYRP1)	melanogenic enzyme	7250.1 ± 1930.9	7713.1 ± 914.7
dopachrome tautomerase (DCT)		1922.6 ± 1187.3	1209.8 ± 280.5
osteopetrosis associated transmembrane protein 1 (OSTM1)	pheomelanin synthesis	105.4 ± 8.0	128.3 ± 62.2

The average FPKM for HEM-L and HEM-D libraries was calculated for melanocyte specific genes involved in pigmentation, classified according to their predicted function. The predicted melanosomal transporter SLC45A2, shown in bold, is the only gene expressed at significantly different levels in HEM-L vs. HEM-D.

Table 7

Expression of cell signaling isoforms in HEM-L and HEM-D.

A. Isoforms only expressed in HEM-D

Gene Name (Gene ID)	Accession Number	Gene Family	Transcript Variant
5-hydroxytryptamine (serotonin) receptor 7 (HTR7)	NM_019859.3	GPCR	Variant D Canonical sequence
frizzled class receptor 6 (FZD6)	NM_001164616.1	GPCR	Variant 3 32aa missing
activin A receptor, type IC (ACVR1C)	NM_145259.2	RK	Variant 1 Canonical sequence
histone linker H1 domain, spermatid-specific 1 (HILS1)	NR_024192.1	TF	Variant 2 Non-coding
zinc finger protein 83 (ZNF83)	NM_001105550.1	TF	Variant 3 Different 5'UTR
nuclear receptor subfamily 6, group A, member 1 (NR6A1)	NM_001489.4	NR	Variant 2 5aa missing

B. Isoforms only expressed in HEM-L

Gene Name (Gene ID)	Accession Number	Gene Family	Transcript Variant
histamine receptor H4 (HRH4)	NM_001143828.1	GPCR	Variant 2 88aa missing
regulator of G-protein signaling 3 (RGS3)	NM_001282922.1	GAP	Variant 2 398aa missing
postmeiotic segregation increased 1(PMS1)	NM_001128143.1	TF	Variant 2 39aa missing
ets variant 7 (ETV7)	NM_001207038.1	TF	Variant 5 77aa missing
T-box 3 (TBX3)	NM_016569.3	TF	Variant 2 Canonical sequence
hes family bHLH transcription factor 6 (HES6)	NM_018645.4	TF	Variant 1 Canonical sequence
cut-like homeobox 1 (CUX1)	NM_001202546.1	TF	Variant 7 39aa missing
myoneurin (MYNN)	NM_001185119.1	TF	Variant 3 29aa missing
WD repeat & HMG-box DNA binding protein 1 (WDHD1)	NM_001008396.2	TF	Variant 2 123aa missing
zinc finger protein (ZNF200)	NM_198087.2	TF	Variant 3 1aa missing
zinc finger protein (ZNF662)	NM_207404.3	TF	Variant 1 Canonical sequence
zinc finger protein (ZNF674)	NM_001039891.2	TF	Variant 1 Canonical sequence
zinc finger protein 75D (ZNF75D)	NM_001185063.2	TF	Variant 2 95aa missing
zinc finger protein (ZNF805)	NM_001145078.1	TF	Variant 2 133aa missing

Transcript isoforms from cell signaling gene families expressed exclusively in lightly pigmented (A) or darkly pigmented (B) HEMs Isoforms below expression threshold (FPKM < 0.1) and with high variability (SEM > 35% of its average FPKM) were excluded. Abbreviations: GPCR, G protein-coupled receptor; RK, receptor kinase; GAP, GTPase-accelerating protein; TF, transcription factor; NR, Nuclear receptor.